Parallel evolution in an invasive plant species:

Evolutionary changes in allocation to growth, defense, competitive ability and regrowth of invasive *Jacobaea vulgaris*

Tiantian Lin

林恬恬

Universiteit Leiden

Lin, Tiantian

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	Prof. Dr. J. Joshi (Universität Potsdam, Germany)
	Dr. M. Macel (University of Tübingen, Germany)

Contents

With my love to my parents and my whole family,

This book is dedicated to my dear grandfather Zhiguang Lin who supported me to pursue my scientific career and will be always alive in my memory.

Chapter 1

General introduction

Invasive species

Invasive species are defined as non-native species that successfully establish and spread when introduced beyond their native range and become extraordinarily prominent in their new habitats (Williamson 1996; Pysek et al. 2004). For the last hundreds of years, with an increase in human travel intensity, a large number of plants, animals, and other organisms have been introduced into new environments around the world by human activity either on purpose, for medicinal, nutritious and horticultural value, or by accident, for example in ballast water on ships (Cassey et al. 2005). These introductions beyond the natural range of potential dispersal aided by human transport are commonly referred as biological invasions. More recently, the pace of this process has increased with modern trade, travel, and technology, so that biological invasions have become a consequence of globalization. One of the corollaries of invasiveness is disrupting local ecosystems, causing reductions in the abundance of native species, interfering with agricultural production and may have a significant influence on human health (Mack et al. 2000). Therefore invasive species are regarded as one of the greatest threats to global biodiversity and cause severe economic losses (Hobbs and Mooney 1998; Kark and Antonio 2002; Pimentel et al. 2005; Pejchar and Mooney 2009; Pyšek and Richardson 2010). It is estimated that approximately 50,000 alien-invasive species have been introduced which cause major environmental damages and losses adding up to almost $120 billion per year in the United States only (Pimentel et al. 2005).

In this study I focus on invasive plant species. They can receive a pest status due to their fast growth and reproduction, and lead to a loss of native biodiversity due to competition and causing negatively ecological and economic impacts on their introduced areas (Rejmánek and Richardson 1996; Manchester and Bullock 2000; Mooney and Cleland 2001; Daehler et al. 2004; Pyšek et al. 2012). Some invasive plant species even produce poisonous compounds which pose a threat to livestock and human health (Deinzer et al. 1977; Wardle 1987; Prakash et al. 1999; Xiao-yu et al. 2004). For example, common ragweed (*Ambrosia artemisiifolia*) originated from North America was introduced incidentally into Europe and is nowadays presenting a major health problem because of its highly allergenic pollen. Furthermore ragweed is estimated to reduce corn crop yield by 55% (Makra et al. 2005). Kudzu (*Pueraria lobata*) was introduced in the United States for erosion control is now a pest species threatening native ecosystems due to its rapid growth rate (Forseth and Innis

2004). Another example is common ragwort (*Jacobaea vulgaris*), which result in four million dollar of annual costs due to livestock losses, decreased pasture yields and increased management costs in Australia (McLaren and Mickan 1996).

Among those plant species which were introduced into new habitats, only 2% of them became invasive weeds although they are not necessarily a pest species in their native areas (Williamson 1996; Williamson and Fitter 1996; Reichard and White 2001; Bell et al. 2003). Knowledge of what makes a species become invasive has a great importance for predicting potentially invasive species before and after introduction and controlling existing invasive species. In addition, invasive species also provide evolutionary biologists and ecologists with the opportunity to study basic long term evolutionary processes by considering invasions as long-term large-scale experiments where major changes in selective forces have occurred (Allendorf and Lundquist 2003).

In relation to evolutionary changes it is worthwhile to point out that, after introduction, genetic variation of an invasive plant species was initially assumed to be reduced by genetic bottlenecks and founder effects (Marshall and Brown 1981; Dlugosch and Parker 2008). However, studies have shown that invasive plant species can have similar or even increased amounts of genetic variation than their native conspecifics due to multiple introductions, hybridization and the release of epistatic genetic variation (Ellstrand and Schierenbeck 2000; Dlugosch and Parker 2008; Doorduin et al. 2010). Such high genetic diversity could allow invasive plants to evolve a certain shift in allocation patterns in response to the novel selection condition, which in turn, could contribute to their invasion success (Baker 1965; Dlugosch and Parker 2008).

Theories on why plants become invasive

Although research on biological invasions has grown rapidly over the past few decades, there is still a lot of debate about why species become invasive (Chun et al. 2010). Some theories attribute plant invasiveness to abiotic factors such as local ecosystem type and climatic condition while others focus more on biotic factors (Sakai et al. 2001; Joshi and Vrieling 2005; Richardson et al. 2007; Colomer-Ventura et al. 2015). In this thesis I will focused mainly on biotic factors. One of the most striking changes in biotic factors after plant invasion is a shift in the herbivore guild. In the native area both specialist herbivores and generalist herbivores are present but upon introduction in the invasive range the herbivores are not co-introduced and the invasive plant species therefore is attacked only by generalist herbivores in the invasive areas. In few cases introduced species are attacked by specialist of congeneric plants species (Castells et al. 2013). The escape from specialist herbivores is at the heart of a suite of hypotheses for explaining the invasive success of alien plant species. A number of hypotheses have been put forward which are centered around changes in herbivore pressure (Jeschke 2014).

One of the most commonly accepted and straightforward hypothesis attributes the increased abundance and vigor of many invasive plant species in their introduced range to the absence of their co-evolved natural enemies, which was redefined by Keane and Crawley (2002) as the Enemy Release Hypothesis (ERH) but originally developed by Williamson

(1996). More specifically, the ERH states that invasive species are freed from their native specialist herbivores though they are still may suffer from herbivore pressure by generalist herbivores and occasional specialist herbivores of congeneric plant species after invasion (Frick 1972; Castells et al. 2013) but the overall level of herbivory is assumed to be much lower than in the native range (Memmott et al. 2000). The reduced loss to herbivory gives the invaders an advantage over the local species leading to an increase in their distribution and abundance (Liu and Stiling 2006).

Additionally, Callaway and Ridenour (2004) proposed the novel weapon hypothesis (NWH) stating that invasive plants possess allelopathic chemicals that are relatively ineffective against their native (adapted) neighbors but are highly inhibitory to newly encountered plants in the invasive range. Later on this hypothesis has been extended to the use of such novel phytochemicals by invasive plants to defend against local herbivores (Cappuccino and Carpenter 2005; Schaffner et al. 2011). In contrast, the biotic resistance hypothesis (BRH) argues that the local herbivores in the introduced ranges could suppress plant invasion since the invasive plants are naive and have not been selected to defend themselves effectively against those herbivores (Elton 1958). However, none of the mentioned hypotheses takes the evolutionary consequence of the shift in enemy guild on plant development into account.

Since plant defense can be costly (Koricheva 2002; Strauss et al. 2002) and many defensive traits are genetically controlled (Fritz and Simms 1992; Mole 1994; Strauss and Agrawal 1999), a reduction in herbivore pressure is expected to exert altered selection on invasive plants that in turn leads to an evolutionary decrease in decreased investment in anti-herbivore strategies during invasion (Colautti et al. 2004). Resources can be saved from defense and allocated to plant growth, resulting in a higher competitive ability and leading to a higher seed reproduction as proposed in the Evolution of Increased Competitive Ability hypothesis (Blossey and Nötzold 1995). So far numerous studies have tested the EICA hypothesis but results are inconsistent (Hobbs and Atkins 1991; Willis et al. 1999; Peltzer and Köchy 2001; Leger and Rice 2003; Bossdorf et al. 2004a; Vila and Weiner 2004; Bossdorf et al. 2005; Zou et al. 2008; Felker-Quinn et al. 2013). Partly this inconsistency is due to differences in the experimental setups, such as the choice of competitors and the choice of herbivores or the parameters used to estimate competitive ability (Bossdorf et al. 2005). Especially the presence or absence of specialist herbivores in different studies can be a very important factor. In addition plant tolerance to herbivory has been largely overlooked as part of a quantitative defense strategy. Therefore more comprehensive studies on competition under herbivore regimes including different type of herbivores that also allow for measuring growth and tolerance are still needed for testing the EICA hypothesis.

It is noteworthy that while invasive species are freed from their specialist herbivores they still are under attack by generalist herbivores in the invasive range (Agrawal and Kotanen 2003; Siemann and Rogers 2003; Parker et al. 2006). Therefore as an extension to the Evolution of Increased Competitive Ability hypothesis, the Shifting Defense Hypothesis takes into account the different selective pressures of specialist and generalist herbivores (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011). It hypothesizes that the shift in the herbivore composition towards a guild which is dominated by generalists in the introduced range is expected to select invasive plants with lower

investment in their total defense by reducing the costly quantitative defences against specialist herbivores and increasing the much cheaper qualitative defences targeted at local generalist herbivores without having the side effect of attracting the specialist herbivores (Feeny 1976; Rhoades and Cates 1976; van der Meijden 1996). As a result a net gain can be saved for additional growth and resulting in an increased competitive ability. From the literatures only a few studies so far have measured the quantitative defenses or the qualitative defenses in the light of SDH and results are still controversial (Doorduin and Vrieling 2011; Wolf et al. 2011; Huberty et al. 2014). Therefore in this thesis I will test the EICA and SDH hypotheses further to study the evolutionary changes in allocation to growth, competitive ability and anti-herbivore defenses of invasive plants.

Growth

In line with the EICA and ERH hypotheses, several invasive plant species have been observed to have higher growth rates compared to their conspecifics in the native ranges (Pattison et al. 1998; DeWalt et al. 2004; Jakobs et al. 2004; Zou et al. 2008; Feng et al. 2009; van Kleunen et al. 2010). Higher growth is expected to be accompanied by a change in underlying factors such as a higher specific leaf area (SLA, the ratio of total leaf area and leaf dry mass) and leaf mass fraction (LMF, the ratio of leaf dry mass and total dry mass) (Poorter 1999), an increased photosynthesis (Shipley 2006) and increased shoot to root ratio (Johnson and Thornley 1987). Poorter & Evans (1998) found that plants could increase light capture as well as their photosynthetic nitrogen use efficiency (PNUE, the rate of photosynthetic capacity per unit nitrogen) by increasing their specific leaf area. Several invasive plant species indeed have been observed to have a higher photosynthesis, SLA and PNUE compare to their native conspecifics which contribute to an increased growth (Durand and Goldstein 2001; McDowell 2002; Onoda et al. 2004; Nagel et al. 2005; Feng et al. 2009; Feng et al. 2011).

Quantitative defenses and qualitative defense

Defense is often divided into two types related to its effect on herbivores: quantitative defenses and qualitative defenses (Feeny 1976; Rhoades and Cates 1976). Quantitative defenses (e.g. structural defense and tannins) are digestibility reducers act against both specialist and generalist herbivores but are costly to produce (Poorter and De Jong 1999). Structural defenses are based on anatomical or structural traits such as leaf thickness, leaf toughness and thorns, which serve as plant primarily defense mechanism that herbivores will first encounter. Qualitative defenses (e.g. alkaloids and glucosinolates) are toxins or deterrents act against generalist herbivores which are relatively cheap to produce, but specialist herbivores are often adapted to these defenses (Berryman 1988; Strauss et al. 2002). Moreover, high levels of such qualitative defenses can even be exploited by the specialist herbivores as an oviposition, feeding stimulant or they can sequester them for their own defense against their natural enemies (Hay et al. 1990; van der Meijden 1996; Müller et al. 2001; Bernays et al. 2003; Macel and Vrieling 2003; Nieminen et al. 2003). Therefore in the native ranges where the specialist and generalist both are present, plants could maintain their quantitative defenses at an intermediate level due to the opposing selective forces from adapted specialist and unadapted generalists herbivores following the

Specialist-Generalist dilemma (van der Meijden 1996). Being introduced into a new habitat where specialist herbivores are largely absent, plants also encounter new assemblages of generalist herbivores which could also exert selection on them. The level of attack by local generalists can vary and some generalist herbivores can even prevent the invasion success of certain plants (Maron and Vilà 2001). In order to efficiently deter local generalist herbivores, qualitative defenses can be increased in the invasive plants while costly quantitative defenses are reduced without the side effect of attracting specialist herbivores. As a result, a net allocation gain can be saved for allocation to growth and reproduction which contributes to an increased competitive ability in invasive plants as predicted by SDH (Doorduin and Vrieling 2011).

Tolerance

Instead of deterring herbivores, plants can also reduce the negative fitness effects of herbivores by mitigating the negative effects of herbivory, in other words by being tolerant to damage (van der Meijden et al. 1988). Tolerance is defined as the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores (Agrawal et al. 1999; Strauss and Agrawal 1999; Fornoni 2011). Tolerance is often considered as a last resort against specialist herbivores which have broken through other defences. It is a costly strategy because leaf tissue is lost through herbivory and reserves for tolerance cannot be used for growth (Bossdorf et al. 2004b).

The mechanism of plant tolerance to herbivore damage is often associated with compensatory regrowth, reallocation of resources and the utilization of storage reserves (Fornoni 2011; Li et al. 2012). Plant tolerance is considered to be costly because tissue is lost through herbivory and perhaps even more importantly for fast growing plants, reserves allocated for tolerance cannot be used for growth (Bossdorf et al. 2004b). Many plant species were recorded to tolerate tissue loss to herbivory through compensatory regrowth (McNaughton 1983; Simons and Johnston 1999). Therefore in this study we focus on regrowth ability and consider it as a common tolerance strategy. It is assumed that resources needed for regrowth are drawn from storage in plant parts that are relatively free from herbivore attack such as roots (Utsumi & Ohgushi 2007). Root carbohydrate storage can be allocated to the regeneration of new leaf tissues during regrowth and the large size of root systems are speculated to represent high levels of storage in many species (Donaghyē and Fulkersoń 1998; van der Meijden et al. 2000; Sosnová and Klimešová 2009; Chen et al. 2013; McCormick et al. 2013; Aranjuelo et al. 2015). Therefore several studies used the root-shoot ratio as a proxy for plant regrowth ability (van der Meijden et al. 1988; Marschener 1998; Wise and Abrahamson 2005; Hochwender et al. 2012). However, the root system has multiple functions and roots can be used by plants for maintaining structure, retrieving nutrients and water for growth and for storing resources. Hence, a large root to shoot ratio may be the result of high storage levels but can also be the consequence of low nutrient availability (Brouwer 1983; van der Meijden et al. 2000). Therefore it is essential to study both the root storage and root size to understand regrowth capacity and the role of the size of the roots for regrowth.

Since storage of resources for regrowth, growth and investment in defense are drawing from the same resource pool, they are competing with each other for limited resources (de Jong and van der Meijden 2000). High levels of regrowth were found to be at the cost of higher growth rates because it relies on high levels of storage of resources in roots upon defoliation by leaf feeding specialist herbivores and such storage cannot be used for growth (van der Meijden et al. 1988). Therefore in the introduced range where the specialist herbivores are absent, I expect selection for invasive plants to have lower storage of carbohydrates in the roots and hence poor regrowth ability but higher investment in plant growth (Saengkanuk et al. 2011).

Parallel evolution in the introduced range

According to above hypotheses, invasive plants should be selected for allocation of resources from costly quantitative defenses and regrowth to increase plant growth and cheap qualitative defenses due to the absence of natural enemies in the introduced range. However, so far evidence that changes in the herbivore guild are the selective force for changes in allocation patterns is largely circumstantial and other biotic or abiotic factors (e.g. local environmental conditions and preadaptation) cannot be ruled out as being important (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Colomer-Ventura et al. 2015). We therefore set out to study a system where multiple invasive regions are compared that differ in climatological conditions throughout the entire thesis. Climate conditions have been considered as one of the most important potential selective forces which can exert a dominant control over the natural distribution of plant species (Pearson and Dawson 2003). If the change in the herbivore guild is the main selective force, parallel evolutionary simultaneous changes are expected in each of the geographically and climatologically differing invasive ranges.

Research questions

According to the EICA and SDH hypotheses, the change of herbivore composition toward a guild that is dominated by generalist herbivores in the introduced range is expected to select invasive plant populations with lower investment in their total defense by shifting from the costly quantitative defences and tolerance to cheaper qualitative defences. In turn a better growth ability will be achieved and contribute to an increased competitive ability over their native conspecifics. In addition, such advantage in competitive ability should be strongly depend on the type or level of herbivory since invasive populations are expected to be more resistance to the generalist herbivore and more susceptible to the specialist herbivore. In this thesis I will test the above predictions using *Jacobaea vulgaris* (synonym *Senecio jacobaea*, Common ragwort). Four separate experiments were conducted to compare the changes in different traits between native and invasive *J. vulgaris* populations. I will address the following questions:

1. Do invasive *Jacobaea vulgaris* genotypes have decreased quantitative defenses (structural defenses and regrowth)?
2. Do invasive *Jacobaea vulgaris* genotypes have increased qualitative defense (chemical defense)?
3. Do invasive *Jacobaea vulgaris* genotypes have increased growth and competitive ability in the absence of herbivores?
4. Is the competitive ability of invasive *Jacobaea vulgaris* genotypes more affected by a specialist herbivore and less affected by a generalist herbivore than that of native genotypes?
5. Did evolution select growth, competitive ability, quantitative and qualitative defenses of invasive *Jacobaea vulgaris* genotypes from geographically and climatological distinct regions all towards the predicted directions?

I will study the changes in growth and anti-herbivore traits between native and invasive *J. vulgaris. Jacobaea vulgaris* is independently introduced in multiple invasive regions that differ in climatological conditions. As such, I will be able to evaluate whether all the studied traits evolved in parallel across these different regions following a shared history of release from specialist natural enemies.

Jacobaea vulgaris

Jacobaea vulgaris (synonym *Senecio jacobaea*, Common ragwort) is a monocarpic perennial species which belong to the *Asteraceae* family (Pelser et al. 2004). It has four distinct life history stages: seeds, seedlings, rosettes and flowering plants. After germination in autumn or in spring, the plant forms a rosette in its first growing season. In spring and early summer (May-June) of the next year the flowering stem develops if the plant reaches a certain size, and after flowering (July-September) the plant dies (Van der Meijden and Van der Waals-Kooi 1979). *J. vulgaris* is a self-incompatible plant and is pollinated by insects, mainly bees, wasps (hymenopteran) and flies (dipteran)(Harper and Wood 1957). It produces a large number of seeds which can survive for several years in the soil (Van der Meijden and Van der Waals-Kooi 1979).

Jacobaea vulgaris is native to Eurasia and was introduced into Australia (Harper and Wood 1957), New Zealand (Poole and Cairns 1940), east coast of North America since the 1850s and west coast of North America since 1900s (Harris et al. 1971). The west and east coast of North America are geographically isolated as they form a discontinuous distribution. Doorduin et al. (2010) found that the amount of genetic variation of native *J. vulgaris* populations does not differ from the different invasive ranges, suggesting that introductions from multiple source populations have occurred. Moreover, an assignment analysis indicated that populations from the Northern-west coast of Europe are the most likely source populations.

Jacobaea vulgaris is recorded to contain more than 34 different pyrrolizidine alkaloids (PAs), as constitutive and qualitative defences against herbivores (Witte et al. 1992; Cheng et al. 2011). Since PAs are toxic to horses and cattle, it can result in significant livestock losses due to alkaloid poisoning and decreased pasture yields, it received a pest status in the

introduced range in the USA (Coombs et al. 1996), Australia (McLaren and Mickan 1996) and New Zealand (Fowler et al. 2000). In a common garden experiment, Joshi and Vrieling (2005) found that invasive *J. vulgaris* had on average 90% higher PA concentration (especially the jacobine like PAs) and 37% higher reproductive biomass than genotypes from the native range. Willis et al. (2000) compared 6 invasive *J. vulgaris* populations with 6 native populations and found no difference in the matter of size. Furthermore, other studies found that PAs of ragwort played an important role in plant resistance to several generalist herbivores (van Dam et al. 1995; Macel et al. 2005; Leiss et al. 2009; Wei et al. 2015).

In the invasive ranges the herbivore guilds of *J. vulgaris* have been reported to be mainly dominated by local generalist herbivores (Poole and Cairns 1940; Frick 1972; Stastny et al. 2005). In the native range *J. vulgaris* is attacked by more than 70 specialist as well as generalist herbivores (Harper and Wood 1957). Most damage is caused by two specialist herbivores, cinnabar moth (*Tyria jacobaeae*) and the flea beetle (*Longitarsus jacobaeae*), which cause in total over 75% of all inflicted damage (James et al. 1992; McEvoy and Coombs 1999). Both these specialist herbivores are adapted to PAs and sequester PAs (Aplin and Rothschild 1972; Zoelen and Meijden 1991; Dobler et al. 2000; Macel and Vrieling 2003). In North Western Europe, in coastal populations, *Jacobaea vulgaris* is completely defoliated once in every 2 or 3 years by larvae of *T. jacobaeae* during middle June. After that, the herbivory of *L. jacobaeae* during August which also cause a large loss of the leaves (Dempster 1971; Islam and Crawley 1983; Windig and Vrieling 1996; van der Meijden et al. 2000). Therefore in the native range *J. vulgaris* shows a strong regrowth capacity after defoliation due to the regular defoliation by these specialist herbivores (Islam and Crawley 1983; van der Meijden et al. 1988). In the last few decades, *T. jacobaeae* and *L. jacobaeae* have been introduced as biological control agents into to the invasive ranges (Syrett 1983; McEvoy et al. 1991; McLaren and Mickan 1996). So far no evolutionary adaptations of invasive *J. vulgaris* populations have been observed after the exposure to the *L. jacobaeae* (Rapo et al. 2010).

In a competitor-free, common garden experiment Joshi and Vrieling (2005) found that invasive *J. vulgaris* had a higher vegetative and reproductive biomass than native genotypes. In addition, invasive genotypes were more susceptible to specialist herbivores but were better protected against generalist herbivores, had lower regrowth ability and produced more pyrrolizidine alkaloids compared to native ones. All these findings were consistent with the prediction of the EICA and SDH hypotheses and indicate that evolutionary shifts may enable genotypes from the invasive range to have an increased competitive ability compared to native ones.

Outline of this thesis

To answer the 1st question, the morphological traits related to growth, structural defenses and underlying regrowth trait of invasive and native *J. vulgaris* individuals were compared in chapter 2 to investigate whether invasive *J. vulgaris* populations have been selected with decreased structural defense and regrowth potential but increased growth as a response to the release from specialist natural enemies. In chapter 3 the 2nd question was answered by comparing plant growth and underlying growth traits, the concentration and composition of

pyrrolizidine alkaloids in the leaves and underlying regrowth traits between invasive and native *J. vulgaris* individuals to investigate whether invasive *J. vulgaris* populations have been selected with reduced regrowth potential but increased qualitative defense and growth as a response to the release from specialist natural enemies. In chapter 4 the evolutionary changes in regrowth and growth of invasive *J. vulgaris* populations were further studied with an artificial defoliation. Both the regrowth ability and underlying traits were compared between invasive and native populations. To answer the question 3, the competitive ability of invasive and native *J. vulgaris* genotypes were compared in chapter 5 using an intraspecific competition setup in which plants from invasive and native populations directly compete against each other. In addition, the same competition setup was subjected to herbivory by either a specialist herbivore or a generalist herbivore to test whether different herbivores affect the competitive ability of invasive populations in order to answer the question 4. Regarding to the question 5, plant growth, competitive ability, quantitative and qualitative defenses of invasive populations from multiple introduced regions which are geographically and climatological distinct were compared in each of the corresponding chapters. At the end, the main findings presented in this thesis will be summarized in chapter 6 and a general discussion & conclusion will be presented.

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Chapter 2

Enemies lost: parallel evolution in structural defense and tolerance to herbivory of invasive *Jacobaea vulgaris*

Tiantian Lin[a]*, Leonie Doorduin[a]*, Andries Temme[a], Thijs L. Pons[b],

Gerda E.M. Lamers[c], Niels P.R. Anten[d], Klaas Vrieling[a]

[a] Institute of Biology, Section Plant Ecology and Phytochemistry, Leiden University, P.O. Box 9505, 2300 RA Leiden, The Netherlands

[b] Plant Ecophysiology, Institute of Environmental Biology, , Utrecht University, PP Box 800.84, 3508 TB Utrecht, The Netherlands

[c] Institute of Biology, Section Microscopy Unit, Leiden University, P.O. Box 9505, 2300 RA Leiden, The Netherlands

[d] Centre for Crop Systems Analysis, Wageningen University, P.O. Box 430, 6700 AK, Wageningen, The Netherlands

*Both authors contributed equally to this work

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Abstract

According to the Shifting Defense Hypothesis, invasive plants should trade-off their costly quantitative defense to cheaper qualitative defense and growth due to the lack of natural specialist enemies and the presence of generalist enemies in the introduced areas. Several studies showed that plant genotypes from the invasive areas had a better qualitative defense than genotypes from the native area but only a few studies have focused on the quantitative defenses and tolerance ability. We compared structural defenses, tolerance and growth between invasive and native plant populations from different continents using the model plant *Jacobaea vulgaris*. We examined several microscopical structure traits, toughness, amount of cell wall proteins, growth and root-shoot ratio, which is a proxy for tolerance. The results show that invasive *J. vulgaris* have thinner leaves, lower leaf mass area, lower leaf cell wall protein contents and a lower root-shoot ratio than native genotypes. It indicates that invasive genotypes have poorer structural defense and tolerance to herbivory but potentially higher growth compared to native genotypes. These findings are in line with the Evolution of Increased Competitive Ability hypothesis and Shifting Defense Hypothesis. We also show that the invasiveness of this species in three geographically separated regions is consistently associated with the loss of parts of its quantitative defense and tolerance ability. The simultaneous change in quantitative defense and tolerance of the same magnitude and direction in the three invasive regions can be explained by parallel evolution. We argue that such parallel evolution might be attributed to the absence of natural enemies rather than adaptation to local abiotic factors, since climate conditions among these three regions were different. Understanding such evolutionary changes helps to understand why plant species become invasive and might be important for biological control.

Keywords

Plant defense; Cell walls; Leaf mass area; Microscopy; Toughness; Invasion ecology; Plant-insect interactions; Biological control.

Introduction

Invasive species are defined as non-native species that successfully establish and spread when introduced beyond their native range (Williamson 1996; Pysek et al. 2004). The spread of invasive species often poses serious threats to the native biodiversity, the ecosystem services of the invaded area, the local agricultural productivity, and human health. Invasive species therefore cause significant environmental damage and economic losses (Hobbs and Mooney 1998; Kark and Antonio 2002; Pimentel et al. 2005; Pejchar and Mooney 2009; Pyšek and Richardson 2010). Only 2% of introduced plant species eventually become invasive weeds although they are not necessarily pest species in their native areas (Williamson 1996; Williamson and Fitter 1996; Reichard and White 2001; Bell et al. 2003). Knowledge of what makes species become invasive has a great importance for predicting potentially invasive species before and after introduction and controlling existing invasive species.

The first theories on why plants become invasive emphasize the absence of natural enemies after introduction of plants in a new area (Williamson 1996; Keane and Crawley 2002; Mitchell and Power 2003)When plants are introduced into a new habitat, they leave their co-evolved natural enemies behind. This release from detrimental herbivore pressure potentially resulting in a fast increase in distribution and abundance (the enemy release hypothesis (ERH), (Keane and Crawley 2002). Further hypotheses were proposed on the basis of evolutionary changes during invasion. The Evolution of Increased Competitive Ability hypothesis (EICA) predicts that the escape from specialist herbivores leads to an evolutionary change in invasive plants in energy allocation from defense to growth which can give invasive plants a higher competitive ability to outcompete local plant species (Blossey and Nötzold 1995).

Defense is often divided into two types related to its effect on herbivores: quantitative defenses and qualitative defenses (Feeny 1976; Rhoades and Cates 1976). Quantitative defenses (e.g. tannins and trichomes) act against specialist as well as generalist herbivores and are costly to produce (Poorter and De Jong 1999). Qualitative defenses (toxins such as alkaloids and glucosinolates) act against generalist herbivores and are cheaper to produce, but specialist herbivores are often adapted to these defenses (Berryman 1988; Strauss et al. 2002). Specialist herbivores often use these compounds as a cue to locate their host plant, as an oviposition and feeding stimulant, and may sequester the qualitative plant defense compounds for their own defense (Hay et al. 1990; van der Meijden 1996; Müller et al. 2001; Bernays et al. 2003; Macel and Vrieling 2003; Nieminen et al. 2003). In the invasive area where specialist herbivores are absent, plant can shift their allocation to produce more of the cheap qualitative defenses against generalist herbivores without having the side effect of attracting the specialist herbivores. By doing so they can decrease their costly quantitative defenses against the absent specialist herbivores. As a net result the plants in the invasive area are well defended and resources can be saved for growth and reproduction which can give these plants a competitive edge over the local plant species (Doorduin and Vrieling 2011). This evolutionary shift of quantitative defense to qualitative defense in the invasive area is called the Shifting Defense Hypothesis (SDH) (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011).

To study the role of the defense mechanisms involved in the evolutionary shift of invasive plant species, most studies have focused on the qualitative defenses while only a few of them have examined quantitative defenses (Rogers and Siemann 2005; Zou et al. 2008; Feng et al. 2009; Alba et al. 2011; Gard et al. 2012; Zhang et al. 2012). Quantitative defenses can be based on morphology or on chemical compounds that occur in higher concentrations (Feeny 1976; Rhoades and Cates 1976). As an important part of quantitative defenses, morphological defenses are based on anatomical or structural traits, such as leaf structures (see Fig. 3), leaf toughness, hairiness and thorns. Such traits confer a fitness advantage to the plant by directly deterring both generalist and specialist herbivores from feeding on it but tend to be costly in terms of resources (Hanley et al. 2007; Kurokawa and Nakashizuka 2008). Several invasive species were recorded to have evolved lower amounts of quantitative defenses after invasion (Willis and Blossey 1999; Siemann and Rogers 2001; Feng et al. 2009; Huang et al. 2010; Doorduin and Vrieling 2011).

Plant cell walls contain several kinds of proteins, such as hydroxyl-proline-rich glycoproteins, proline-rich proteins and glycine-rich proteins, which play a role in defense, growth, development, signaling, intercellular communication and environmental sensing (Showalter 1993). Besides protein, plant cell walls also consist of carbohydrates, lignin, water, and encrusting substances which could serve as part of the structural defenses as well (Bradley et al. 1992; Showalter 1993). Feng et al. (2009) found that invasive *Ageratina adenophora* had 45% lower cell wall protein content than native populations which resulted in a poorer structural defense. Another important structural defense is leaf toughness. It can reduce the suitability of leaves as a food source for herbivores through indigestible polymers such as cellulose and lignin in plant tissues (Raupp 1985). Furthermore, leaf hardness is a defense property which acts as a physical barrier to normal feeding or oviposition by phytophagous insect herbivores (Wright et al. 1989; Kogan 1994). In studies on leaf morphological traits leaf hardness was found to be strongly positively correlated with leaf mass per area (the ratio between the dry mass and the area of plant leaves, LMA). Therefore LMA is used as an easy-to-assess index of structural biomass (Groom and Lamont 1999; Wright and Cannon 2001; Hanley et al. 2007). Moreover, the reciprocal of LMA, the specific leaf area (SLA), is often associated with plant growth rates (Castro-Díez et al. 2000). It is argued that a lower LMA/higher SLA is one of the most important traits associated with high relative growth rate, small seed mass, and invasiveness (Grotkopp and Rejmánek 2007; Hanley et al. 2007; Feng et al. 2008). Several invasive plant species were recorded to have lower LMA/higher SLA than their native congeners (Baruch and Goldstein 1999; Durand and Goldstein 2001; Nagel and Griffin 2001; Burns 2006; Feng et al. 2008). According to above, cell wall proteins, the mechanical properties of cell walls as well as leaf toughness and LMA can be considered as important traits for evaluating plant structural defenses.

Instead of deterring herbivores, plants can also reduce the negative fitness effects of herbivores through being tolerant to damage (van der Meijden et al. 1988). Tolerance is defined as the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores (Agrawal et al. 1999; Strauss and Agrawal 1999). A variety of plants suffer from high levels of herbivory or even frequent defoliation during their life time. In order to survive from such attacks, some plant species are likely to develop compensating mechanism: regrowth (van der Meijden et al. 1988). As one of the most common tolerance

strategies of plants, regrowth ability implies the saving and storage of nutrients and energy in plant parts that are relatively free from herbivore attack (McNaughton 1983; Rosenthal and Kotanen 1994; de Jong and van Der Meijden 2000; Anten and Pierik 2010). Depending on the sink–source relationships, the reallocation of resources in different plant organs could result in a compensatory regrowth to replace damaged tissue after herbivory (Utsumi and Ohgushi 2007). Since root-shoot ratio was found to be directly correlated with regrowth in *Jacobaea vulgaris* (van der Meijden et al. 1988), we considered the root-shoot ratio as a proper proxy for tolerance to herbivory in this study. Although structural defenses and tolerance play such significant roles against herbivore attack, they have been rarely studied together in the light of the EICA and SDH hypothesis.

It is worth to point out that so far evidence that the escape from specialist herbivores is the selective force leading to changes in allocation patterns in invasive plants is largely circumstantial and other biotic or abiotic factors can also play important roles. We therefore set out a study system where multiple invasive regions are compared that differ in climatological conditions. If the change in the herbivore guild is the main selective force, parallel evolutionary changes in traits related to tolerance, structural defenses and growth are expected in each of the geographically and climatologically differing invasive ranges.

In this study we ask if the invasive plant genotypes have evolved decreased amounts of structural defenses and tolerance compared to the plant genotypes from the native area. We examined the microscopical structures of leaf traits, the amount of cell wall proteins, leaf toughness, leaf thickness (LMA) and tolerance (root-shoot ratio) in native and invasive plant genotypes of common ragwort, *Jacobaea vulgaris*, our model plant. We compared native and invasive *J. vulgaris* genotypes and expect invasive genotypes to have (1) lower structural defense against herbivory (2) have a decreased root-shoot ratio which leads to a lower tolerance and regrowth ability. Moreover, we evaluated these traits in introduced populations from geographically and climatically distinct invaded regions (New Zealand, Australia, and North America). As such, we were able to evaluate whether structural defense and tolerance evolved in parallel across these different locales following a shared history of release from specialist natural enemies. To our knowledge, this study is one of the first that focuses on the comparison of both the tolerance and structural defense between the same plant species from invasive and native areas. Hence the result would contribute to the critical evaluation of the role of tolerance and structural defense involved in the evolutionary shift mechanism behind invasion success.

Material and methods

Study species

Jacobaea vulgaris formerly known as *Senecio jacobaea*, is a monocarpic perennial plant that belongs to the family of the *Asteraceae*. It is native to Eurasia and was introduced into parts of New Zealand (first recorded at 1874) (Poole and Cairns 1940), Australia (first recorded at 1890)(McLaren et al. 2000), and North America (first recorded at 1913) (Harris et al. 1971). In the native range it is attacked by more than 70 herbivores and most herbivory is by the two main specialist herbivores: *Tyria jacobaeae* (Cinnabar month) and *Longitarsus*

jacobaeae (Fleabeetle) (Joshi and Vrieling 2005). In the introduced ranges, *J. vulgaris* was recorded to be fed by more than 40 species of generalist arthropods in North America but no specialists herbivore was observed (Frick 1972). *Jacobaea vulgaris* contains pyrrolizidine alkaloids (PAs) which are toxic to horses and cattle (Johnson et al. 1985; Stegelmeier et al. 1999; Gardner et al. 2006). Therefore it received a pest status because infestations have resulted in significant livestock losses due to alkaloid poisoning and decreased pasture yields (Coombs et al. 1996). Because of its weediness and toxicity, it has been intensively studied to discover how selection has changed these traits in the invasive range (Willis et al. 2000; Joshi and Vrieling 2005; Stastny et al. 2005). PA's act as oviposition and feeding stimulants for the specialist herbivore *T. jacobaeae* (Macel et al. 2004; Potter et al. 2004) and both *T. jacobaeae* and *L. jacobaeae* sequester PAs for their own defense against predators (Aplin and Rothschild 1972; Zoelen and van der Meijden 1991; Dobler et al. 2000). In a common garden experiment, Joshi and Vrieling (2005) found that invasive *J. vulgaris* had on average 90% more PAs (a qualitative defense) and 37% higher reproductive biomass than genotypes from the native areas. Furthermore invasive *J. vulgaris* were more vulnerable to the specialist herbivores *T. jacobaeae*, *L. jacobaeae* and *Platyptilia isodactylus* but better protected against the generalist herbivores *Mamestra brassicae* and *Spodoptera exigua* (Joshi and Vrieling 2005). These findings are in line with the SDH hypothesis and indicated an evolutionary shift from lower protection against specialist towards increased growth and reproduction as well as higher protection against general herbivores in *J. vulgaris*. Furthermore, *J. vulgaris* in the native area is regularly defoliated by *T. jacobaeae* and shows a strong regrowth after defoliation (Islam and Crawley 1983; van der Meijden et al. 1988).

Recently, *T. jacobaeae, L. jacobaeae* and other specialist herbivores have been introduced into to the invasive areas as biological controls for combating the invasion of *J. vulgaris* for several years (McEvoy et al. 1991; McEvoy and Coombs 1999). However, Rapo et al. (2010) only found small differences between traits of invasive *Jacobaea vulgaris* populations (New Zealand and North America) with and without biological control history of *L. jacobaeae* but larger differences between native and invasive populations. It suggests that the recent introduction of the biological control agents did not yet cause a rapid evolutionary adaptation of *J. vulgaris* populations in the introduced range towards the native phenotype.

Further, Doorduin et al. (2010) found that the amount of neutral genetic variation of *J. vulgaris* in the invasive habitats was equal to the native habitat. This suggests that multiple introductions from different source populations have occurred as the native populations are significantly different from each other with respect to neutral genetic variation. This indicates that the filtered introduction of pre-adapted genotypes is not a likely explanation and that indeed evolutionary changes occurred in the invasive *J. vulgaris* plants at the introduced areas than pre-adaptation. In addition, Joshi and Vrieling (2005) found that the studied traits in the three different invasive areas all showed the same significant trends, a phenomenon not easily explained by pre-adaptation.

Plant material and growth conditions

Seeds were collected from 10-15 plants and bagged individually from 19 native populations in Europe and from 20 invasive populations in New Zealand, Australia and the USA

(Supplementary Table S1 and Supplementary Fig. S1). Seeds were germinated in petri dishes with moistened filter paper and from each population five seedlings from five different maternal lines were potted in 0.5 L pots with 5% potting soil (Slingerland potground, Zoeterwoude, The Netherlands), 95% sandy soil (collected from Meijendel, The Netherlands, 52º13'N, 4º34'E) and 0.75g Osmocote slow release fertilizer (Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA; N:P:K:MgO 15:9:11:2.5). Plants were grown in a climate room for 17 weeks at 20°C, 70% humidity, 16 hours daylight with a light intensity of 113 umol PAR $m^{-2}s^{-1}$. They were watered when needed. After 10 weeks 50 mL Pokon solution NPK 7-5-6 (8 $mL \cdot L^{-1}$) and Fe-EDTA of 3.2 $g \cdot L^{-1}$was given to the plants twice a week. At the end, two genotypes per population were randomly picked to be used for the microscopic analysis (after 14 weeks), for toughness measurements (after 16 weeks) and for cell wall measurements one of these two genotypes were randomly chosen (after 12 weeks). After 17 weeks all of the five genotypes per population were harvested for dry weight (table 1). For practical reasons the number of replicates per population for microscopic analysis, toughness measurements and cell wall measurements are low (1 or 2). However as we are not interested in differences between populations within a range but to differences between ranges we chose to have a broad sampling of populations to estimate differences between ranges.

Structural defenses

Leaf microscopic measurements

For microscopic measurements, sections were cut from the tip of the middlemost leaf of each plant after 14 weeks of growth using a hand microtome (Fig. 1). Sections were then stained using propidium iodide for 15 minutes. Propidium iodide stains DNA as well as cell wall material. Images were acquired by using a Zeiss LSM exciter on an Axio observer microscope (Exc. with a HeNe 534 laser and em. LP 560). A full cross section of the leaf was obtained by tile scanning the specimen with a 40 x 1.2 NA Plan APO water immersion objective. This gave an image size of 321.43 µm x 482.14 µm with a resolution of 80 nm per pixel. As leaf structure parameters, we measured the upper and lower cuticle thickness and epidermis cell wall thickness, the palisade parenchyma layer thickness, the sponge parenchyma layer thickness and leaf thickness, which are all considered to contribute to leaf toughness and structural defense. Measurements were made using ImageJ® 1.42q and each measurement of cell wall thickness and leaf layers was made 5 times on different parts of the cross-section as outlined in the Fig. 2. In total 14 cell traits were measured as indicated in the Fig. 2. For statistical analysis the average of the five measurements was taken.

Leaf toughness measurements

Leaf toughness was measured at the middlemost leaf of each plant after 16 weeks of growth using a punch and die method on an Instron 4000 according to Onoda et al. (2008) (Fig. 1). A flat ended sharp-edged cylindrical steel punch (diameter=1.345mm) and a steel die with a sharp-edged hole (0.2 mm) were used. The punch and die were installed into a general testing machine (5542, Instron, Canton, MA, USA), and the punch was placed to go through the middle of the hole of the die without any friction. When the punch started to compress the leaf, a sharp increase in force is observed. Maximum force (N) was recorded just before

the leaf fractured. The speed of the punch was constant (24.7 mm min^{-1}) and the machine recorded the load was applied to the sample simultaneously. Work (µ Joule) was also recorded during the whole process and the total work to penetrate the leaf was calculated as the area under the force displacement curve. Punch strength and punch toughness were calculated from a force-displacement curve (Aranwela et al. 1999). Punch strength(MN • m^{-2} or MPa) = Maximum force /A, and punch toughness (kJ • m^{-2}) = work/A, where A is the area of the punch (1.42 mm^2). Leaf mass per area (LMA) was calculated as the ratio between the dry mass and leaf area from the same leaf used for toughness measurements. Each plant was measured two times on the same leaf and for statistical analysis the average of the two measurements was taken.

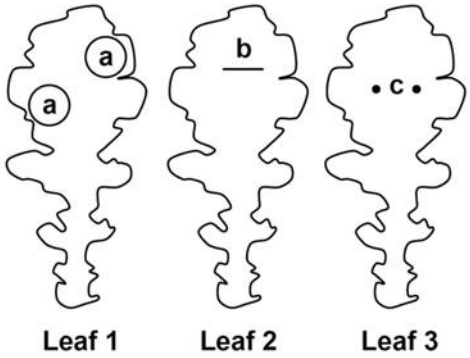

Fig. 1. Locations of the different leaf measurements. Leaf 1: location of the: punches for cell wall material analysis. Leaf 2: Location of the coupes for microscopic measurements. Leaf 3: Location of the spots for toughness measurements.

Cell wall protein measurements

From each population one plant was randomly selected for cell wall proteins extraction using the protein extraction protocol of Takashima et al. (2004). Two 1 cm diameter punches were extracted from the middlemost leaf after 12 weeks of growth, avoiding the main leaf veins (Fig. 1). Water soluble material and SDS soluble material were removed. The remaining cell wall material was oven dried at 60°C for 18 hours and weighed as cell wall proteins. Each plant was measured three times on the same leaf and for statistical analysis the average of three measurements was taken. Therefore each population is only represented by one sample but they represent a random estimate of what is present in each distribution range.

Growth measurements and tolerance

After 17 weeks all plants were harvested, dry weight of shoots and roots were measured after oven drying at 60°C for a minimum of 48 hours. From leaves that were used in the previous measurements, fresh and dry mass were measured and added to the shoot mass. Root-shoot ratio of each plant was calculated and was considered to be trait associated with tolerance.

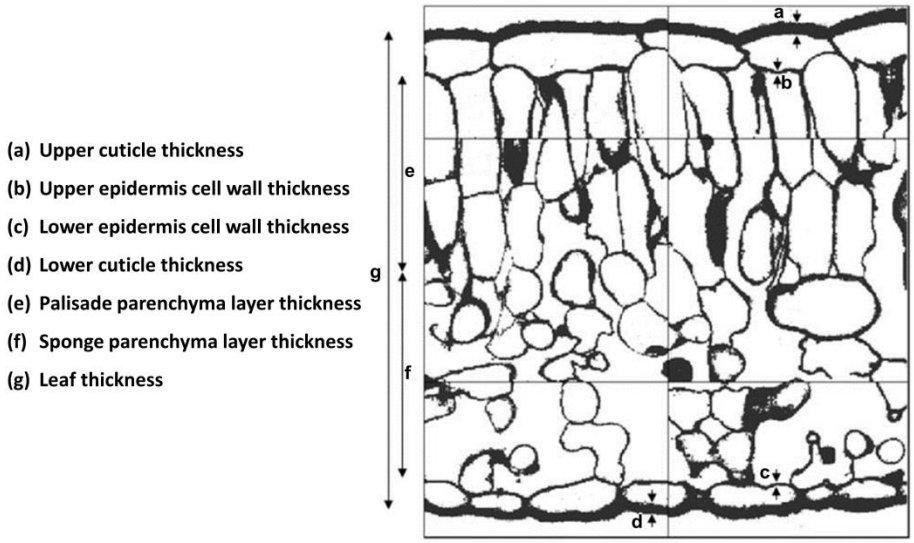

Fig. 2. Measurements made on leaf cross section of native and invasive *J. vulgaris* plants Arrows indicate measurements made. Sample cross section is a cropped, modified and cleaned up version of a coupe from Landsborough, New Zealand sample.

Statistical analysis

As the main interest of this study was to find differences in invasive versus native areas, statistical analysis was performed by a nested ANOVA, with origin and population nested within origin as fixed factors. Normality of the residuals was checked with a Kolmogorov-Smirnov test. For the cell wall protein measurements a one-way ANOVA was performed for analyzing the data since from each population only one plant was measured. Leaf thickness, leaf mass ratio, cell wall protein, shoot mass and root-shoot ratio, were compared between invasive populations from the three geographic regions (North America, New Zealand and Australia) and the native populations. The differences among this four regions were analyzed by a post hoc LSD test. All analyses were carried out using SPSS 18.0 (SPSS: An IBM Company).

To exam the difference in local climate among the four geographic regions (Europe, USA, Australia and New Zealand), 19 bioclimatic variables of the current conditions (ca 1950–2000) at the collected site of each sampled population were downloaded from the WorldClim dataset (http://www.worldclim.org/current) in 5 arc-minutes resolution. A partial least square-discrimination analysis (PLS-DA) was performed with the SIMCA-P software (v.11.0, Umetrics, Umea, Sweden) for classifying all sampled populations based on the 19 bioclimatic variables. The scaling method for PLS-DA was unit-variance and the model was validated by using the permutation method through 20 applications.

Results

Structural defense

Leaf Microscopy

Out of the seven measurements on *J. vulgaris* leaf cross sections, only two showed a difference between native and invasive populations: the lower epidermis cell wall thickness and leaf thickness (Table 1). Lower epidermis cell walls from the invasive origin were 12.1 % thicker (p=0.024) than that of the native populations. The invasive populations had significant thinner leaves than native populations (p=0.023). Furthermore, there was a trend towards a thinner (7.1%) palisade parenchyma layer in the invasive populations (p=0.065).

Table 1. Average leaf microscopical traits in µm of invasive and native *J. vulgaris* populations. Averages were tested with a nested ANOVA, with origin and population nested within origin as two fixed factors.

Measurements (µm)	***Native***	***Invasive***	***P (origins)***	***P (populations)***
Upper cuticle thickness	3.44±0.14	3.49±0.13	NS	NS
Upper epidermis cell wall thickness	1.11±0.04	1.11±0.03	NS	NS
Lower epidermis cell wall thickness	0.99±0.04	1.11±0.03	0.024	NS
Lower cuticle thickness	2.16±0.08	2.28±0.10	NS	NS
Palisade parenchyma layer thickness	119.3±3.6	110.79±3.3	NS (0.065)	NS
Sponge parenchyma layer thickness	120.9±3.7	120.21±3.6	NS	NS
Leaf thickness	276.6±3.0	264.53±3.1	0.023	NS

Values are mean values ± SE (n= 20 for invasive populations and n=19 for native populations).
P (origins): significance level of nested ANOVA between invasive and native origins.
P (populations): significance level of nested ANOVA among populations. NS= not significant.

Leaf toughness, LMA and cell wall proteins

Leaf punch strength and punch toughness did not differ significantly between native and invasive *J. vulgaris* populations (Table 2). LMA was 8.3% lower in the invasive *J. vulgaris* populations compared to the native ones (p=0.038). No significant differences were found among populations. Invasive populations contained on average 10.8% lower amounts of cell wall protein per unit leaf area than the native populations on the basis of leaf area (p=0.037), but there was no significant difference on the basis of leaf mass (Table 3).

Table 2. Average punch strength, punch toughness and LMA of invasive and native *J. vulgaris* populations. Averages were tested with a nested ANOVA, with origin and population nested within origin as two fixed factors.

Measurements	***Native***	***Invasive***	***P (origins)***	***P(populations)***
Punch strength (MPa)	0.636±0.015	0.636±0.012	NS	NS
Punch toughness (kJ • m^{-2})	0.210±0.007	0.227±0.009	NS	NS
LMA(g• m^{-2})	62.74±1.88	57.55±1.50	0.038	NS

Values are mean values ± SE (n= 20 for invasive populations and n=19 for native populations).
P (origins): significance level of nested ANOVA between invasive and native origins.
P (populations): significance level of nested ANOVA among populations. NS= not significant.

Table 3. Average amount of cell wall proteins on the basis of leaf mass and of leaf area of invasive and native *J. vulgaris* populations. Averages were tested with a one-way ANOVA.

Measurements	***Native***	***Invasive***	***P***
Cell wall proteins (g • g^{-1} dry weight)	0.291±0.011	0.278±0.012	NS
Cell wall proteins (g • m^{-2})	10.63±0.25	9.48±0.31	0.037

Values are mean values ± SE (n= 20 for invasive populations and n=19 for native populations)
P: significance level of one-way ANOVA between invasive and native origins. NS= not significant.

Growth traits and tolerance

After 17 weeks of growth, plants from invasive populations had 13.7% more shoot mass (p=0.029) than that of native populations (Table 4). No significant difference (p=0.089) in the root mass was found but there is a tendency that invasive genotypes had smaller roots. Furthermore, the root-shoot ratio of the invasive *J. vulgaris* populations were 18.7% lower than those of the native populations (p=0.030). Furthermore, invasive populations tend to have a higher total biomass than native populations but this difference was not statistically significant. Significant differences were found among the populations for all the growth traits (p=0.002 for shoot mass, p=0.006 for root mass, p=0.003 for shoot mass and p=0.034 for root-shoot ratio, respectively).

Parallel evolution: Comparisons of three invasive regions with native region

The PLS-DA plot showed that the sampled populations of the four geographic regions were clearly separated based on the 19 bioclimatic variables (Fig. 3). It shows the local climate conditions differed between the native and invasive range but also among the three invasive regions.

Table 4. Average shoot dry mass, root dry mass, total plant dry mass and root-shoot ratio of invasive and native *J. vulgaris* populations. Averages were tested with a nested ANOVA, with origin and population nested within origin as two fixed factors.

Measurements	***Native***	***Invasive***	***P (origins)***	***P(populations)***
Shoot mass (g)	6.13±0.25	6.97±0.24	0.029	0.002
Root mass (g)	5.61 ±0.35	5.23±0.31	NS (0.089)	0.006
Total mass (g)	11.74±0.52	12.20±0.49	NS	0.003
Root-shoot ratio ($g \bullet g^{-1}$)	0.922±0.046	0.750±0.037	0.030	0.034

Values are mean values ± SE (n= 20 for invasive populations and n=19 for native populations).
P (origins): significance level of nested ANOVA between invasive and native origins;
P (populations): significance level of nested ANOVA among populations. NS= not significant.

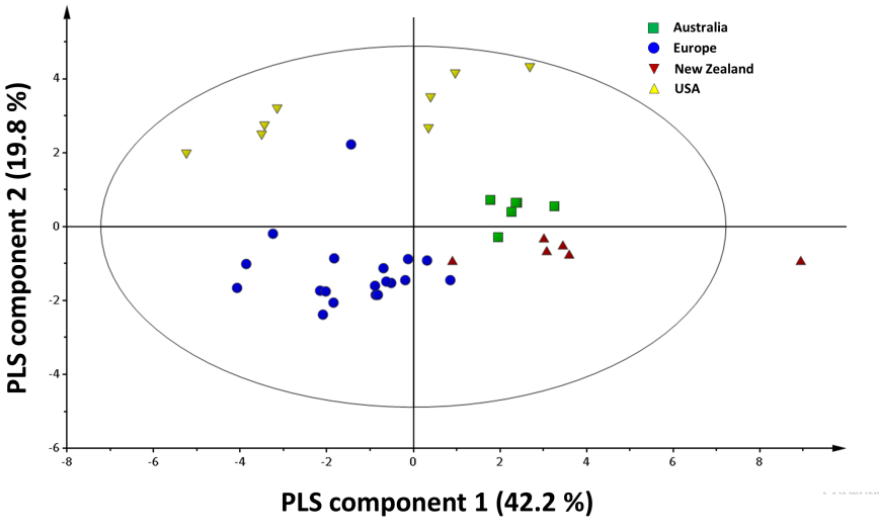

Fig. 3 Partial least square-discrimination analysis (PLS-DA) plot classifying the four geographic regions of native and invasive ragwort based on 19 bioclimatic variables from the collected sites of each sampled population (N=19 for Europe, N= 6 for Australia, N=6 for New Zealand and N=8 for USA).

We further investigated the five traits (leaf thickness, leaf mass ratio, cell wall protein, shoot mass and root-shoot ratio) which are significantly differed between invasive and native genotypes and compared them among invasive populations from three geographic regions (North America, New Zealand and Australia) and native populations (Fig. 4). The results showed that invasive populations from the three regions were all different from native populations in all the traits. Moreover, none of these traits from invasive populations differed significantly among the three geographic regions (ANOVA, root-shoot ratio:, $F_{2, 19}$ =0.265, p=0.768; shoot dry weight: $F_{2, 19}$ =2.578, p=0.082; LMA: $F_{2, 19}$ =0.062, p=0.940; Leaf thickness: $F_{2, 19}$ =0.706, p=0.501; cell wall proteins per leaf area: $F_{2, 19}$ =2.853, p=0.072).

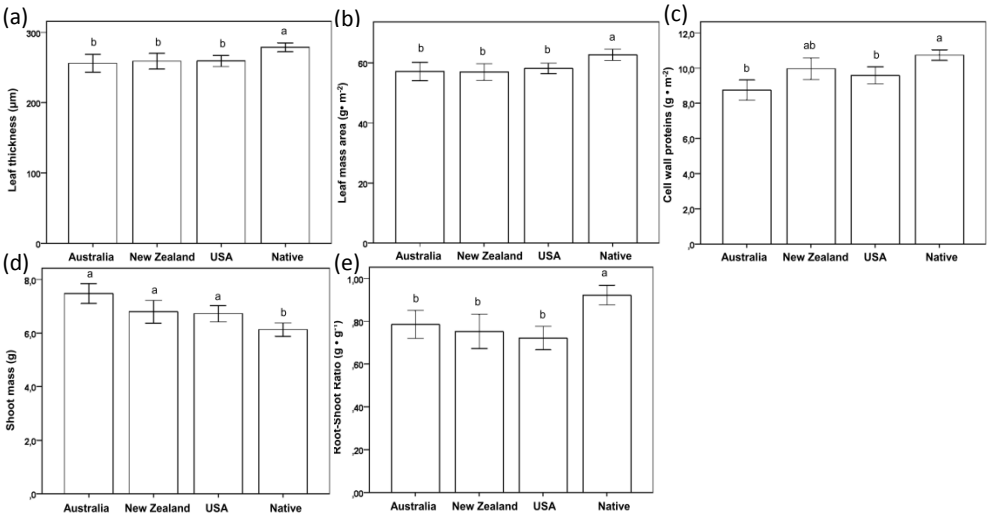

Fig. 4. The comparisons of leaf thickness (a), leaf mass area (b), cell wall protein (c), shoot mass (d) and root-shoot ratio (e) among invasive populations from three geographic regions (Australia, New Zealand and USA) with native populations. Values are means ± SE. Different letters indicate significant differences among treatments at $p<0.05$ according to a post hoc LSD test (ANOVA).

Discussion

Structural defense

According to the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002) plants experience less herbivore pressure when they are introduced into a new habitat and leave behind their old, co-evolved, natural enemies. Escape from specialist herbivores, often requiring costly deterrents to keep them at bay, is thought to allow for an evolutionary shift in energy allocation from defense to growth (Feng et al. 2009; Ru et al. 2011). This shift can give invasive plants increased competitive ability, outcompeting local plant species (Blossey and Nötzold 1995). In this study we did not find strong evidence from microscopic analysis supporting our hypothesis that invasive genotypes have evolved lower structural defense against herbivory. The most interesting anatomical finding was that invasive *J. vulgaris* had significantly thinner leaves. Leaf thickness plays an important role in plant anti-herbivore defense and Peeters (2002) found it was negatively associated with densities of external chewers. Thus leaf thickness could potentially be used as one of the leaf structural traits to predict the functional composition of herbivorous insect assemblages.

Leaf toughness, however, was not different between invasive and native types. We did not find that invasive *J. vulgaris* genotypes had significant lower leaf punch strength and punch toughness than native genotypes. Yet, we did find that the invasive genotypes showed smaller LMA, which is consistent to our hypothesis. Low LMA facilitates efficient light capture and such plants tend to have productive but short-lived leaves and high growth rates (Poorter and Evans 1998). Conversely high LMA is associated with leaf structural traits that confer toughness and thus resistance against herbivores (Hanley et al. 2007). Therefore

a lower LMA could be associated with faster growth of invasive plants which in turn may contribute to their ability to outcompete local plants in the introduced habitats, but this lower LMA may also entail a lower level of structural defense. In this study, we did find that the LMA tended to be positively correlated with punch toughness and strength but this trend was not significant (Pearson correlation one-tailed test, r=0.032, p=0.065 and r=0.022, p=0.107, respectively).

Leaf cell walls constitute a substantial amount of nitrogen and account for 30-50% of leaf dry mass, therefore a large amount of cell walls could increase leaf structural toughness which, in turn, would contribute to a greater tolerance to physical damage (Onoda et al. 2004). In this study we found that the invasive *J. vulgaris* genotypes had a 10.8% lower amount of cell wall proteins per leaf area than the native genotypes, which resulted in a significantly poorer structural defense to herbivory. In addition, Feng et al. (2009) also found invasive *Ageratina adenophora* plants to have lower cell wall protein contents than native populations. They argued that selection for invasiveness could in this species be associated with preferential allocation of nitrogen to photosynthetic functions at the expense of allocation to cell walls, as this reallocation gives the invasive plants a competitive advantage at the cost of a poorer structural defense. Since the specialist natural enemies in the introduced habitats are absent, the invasive plants could gain benefits from such trade-offs between defense and growth for their invasion success.

Growth traits and tolerance

On average there were no significant differences in total mass and root mass between native and invasive *J. vulgaris* genotypes after four weeks of growth. However, we found that invasive *J. vulgaris* genotypes invested more in the aboveground parts than in underground parts resulting in a significantly larger shoot mass and, as already noted, had lower LMA values compared to the native genotypes. Having larger shoots and thinner leaves may enable invasive genotypes to grow faster inherently (Poorter 1999; Lake and Leishman 2004; Leishman and Thomson 2005; Grotkopp and Rejmánek 2007), and, independent of this growth potential, also enable them to compete more effectively for light (Schieving and Poorter 1999). Indeed Joshi and Vrieling (2005) found that invasive *J. vulgaris* plants produced significantly higher biomass and had 37% higher reproductive output compared to native plants in a common garden experiment after eight month growth. Therefore the relatively small and only marginally significant difference in final biomass between native and introduced is probably associated with the short duration of the experiment and the difference would likely have amplified had the experiment been longer.

On the other hand one could also expect that a decreased allocation to the root might lead to an increased allocation to the shoot in order to increase the amount of photosynthetic tissue and/or to be a better competitor for light. Indeed invasive *J. vulgaris* populations had a significantly lower root-shoot ratio (Fig. 4). Furthermore, the root-shoot ratio is also associated with plant regrowth ability after damage. Consistent with our hypothesis, we found that invasive *J. vulgaris* genotypes have lower regrowth ability and tolerance than native genotypes. This is further supported by the finding of Joshi and Vrieling (2005) who found that native *J. vulgaris* genotypes had a 12% higher regrowth ability after full defoliation.

It is worth to point out that there are not many studies specifically focusing on differences plant underground development between native and invasive plants, but among the among the available findings there appears to be no consistent trend towards invasive plants having lower root-shoot ratios (D'Antonio and Mahall 1991; Pattison et al. 1998; Marler et al. 1999; Claridge and Franklin 2002; Wilsey and Polley 2006; Kumschick et al. 2013). Root-shoot ratio can be influenced by variable factors such as soil condition, local competition and herbivory (Monk 1966; Ågren and Ingestad 1987; van der Meijden et al. 1988; Hutchings and John 2004; Poorter et al. 2012). We argue that this allocation-to-root phenomenon we found in the native *J. vulgaris* is species specific, which is due to the selection pressure of its specialist herbivore *Tyria jacobaeae* in the natural habitats. The foliar-feeding larvae of this specialist herbivore can remove all the aboveground parts of *J. vulgaris* plants within a short time period (Dempster 1971). However, the main period of herbivory of this univoltine moth only lasts for six weeks with a peak in June. Therefore native *J. vulgaris* could develop a tolerance strategy in order to survive from such attacks. During the plant's development, resources from aboveground shoots are allocated to underground parts, which resulted in a larger root system for later regrowth after herbivory (Islam and Crawley 1983; van der Meijden et al. 1988).In contrast, it could be argued that the lower root-shoot ratio of invasive *J. vulgaris* genotypes represents a redistribution of resources from root storage (as in native genotypes) to growth of aboveground parts, and thus increasing potential growth.

Parallel evolution

Notably, we also investigated the difference among invasive populations of *J. vulgaris* from three geographic regions (North America, New Zealand and Australia). The results showed that of all traits that significantly differed among invasive and native genotypes, none differed significantly between the three geographic regions (Fig. 4). It showed that the invasive *J. vulgaris* populations from those three geographically separated regions changed in the same direction suggesting a parallel evolution occurred (Joshi and Vrieling 2005). When plants are introduced into a new area, local adaptation to abiotic factors could also exert selective forces on invasive plants during evolution in addition to the absence of specialist herbivores (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Felker-Quinn et al. 2013; Colomer-Ventura et al. 2015). Climate can exert a dominant control over the natural distribution of plant species (Pearson and Dawson 2003). In this study we found the local climate condition differed between the three invasive regions (USA, New Zealand and Australia, Fig. 3). Climatic condition are considered as a potential selection force which in turn might shape the different defensive and growth traits in *J. vulgaris* populations among the three invasive regions. However, we found changes of the same magnitude and direction in quantitative defenses and tolerance in the three invasive regions. The absence of the change in traits correlated with climatic factors suggest that the disappearance of selection pressures from specialist herbivores rather than the adaptation to local abiotic factors caused the parallel changes in quantitative and tolerance traits.

In conclusion, invasive *J. vulgaris* was found to have thinner leaves, lower LMA, lower cell wall proteins contents and smaller root-shoot ratio, resulting in a poorer structural defenses and lower tolerance ability to herbivory but higher potential growth and competitive ability compared to native genotypes. These results support the Evolution of Increased Competitive

Ability hypothesis and Shifting Defense Hypothesis that due to the absence of adapted specialist herbivores, a net gain will be saved by the invasive plants by investing less in structural defense and tolerance for better growth. And all those traits that significantly different among native and invasive genotypes all changed in the same direction as predicted by the SDH in all three geographically separated invasive regions. This is in agreement with a parallel evolution occurred in those three different regions.

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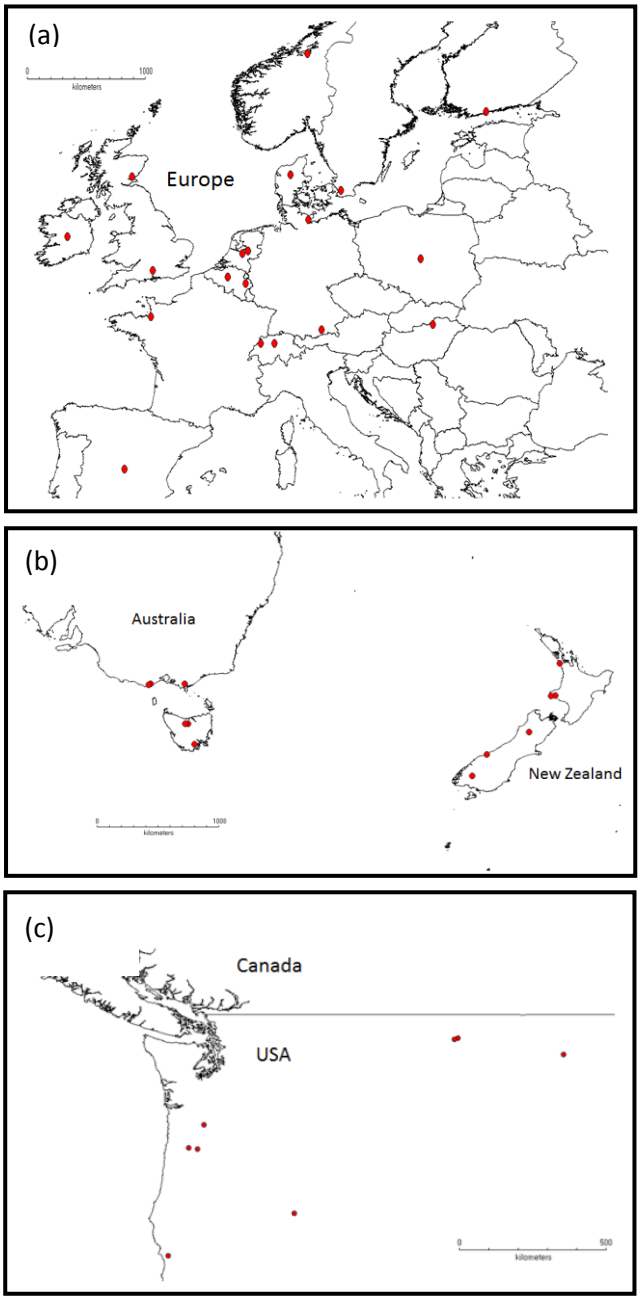
Supplementary Table S1. Origin of *J. vulgaris* populations used in this study and number of plants used for the different measurements. Growth: measurement of shoot and root dry mass after17 weeks of growth. Microsc: measurement of leaf structural parameters after 14 weeks of growth. Tough: measurement of leaf toughness and LMA after 16 weeks of growth. CW: measurement of cell wall proteins after 12 weeks of growth.

					Number of plants used in measurements			
Origin	**Country**	**Location**	**Longitude**	**Latitude**	**Growth 17 weeks**	**Microsc. 14 weeks**	**Tough. 16 weeks**	**CW 12 weeks**
Invasive	*Australia*	Barramunga	143°41' E	38°33' S	4	2	2	1
		Beech forest	143°33' E	38°38' S	4	2	3	1
		Dairy Plains	146°31' E	41°34' S	4	1	1	1
		Franklin	147°00' E	43°05' S	5	2	2	1
		Mayberry	146°18' E	41°33' S	4	2	2	1
		Turton's Creek	146°15' E	38°33' S	5	2	1	1
	New Zealand	Craigieburn	174°13' E	39°25' S	4	2	2	1
		Landsborough	169°02' E	43°53' S	5	2	2	1
		Maruia	172°13' E	42°11' S	2	2	2	1
		Opunake	173°51' E	39°27' S	5	2	2	1
		Southland	167°55' E	45°28' S	1	1	1	1
		Whatipu	174°31' E	37°01' S	4	2	2	1
	USA	Corvallis	123°19'W	44°58' N	5	2	2	1
		Spur Road, Conrad	111°35'W	47°48' N	5	2	2	1
		Six Rivers National Forest	123°57'W	41°42' N	4	2	2	1
		Kootenai National Park	114°53'W	48°17' N	4	2	2	1
		No Bear road	120°00'W	43°00' N	5	2	2	1
		South Cooper montain	122°50'W	45°40' N	4	2	2	1
		Salem	123°02'W	44°56 N	4	2	2	1
		Surprise Hill	115°00'W	48°15'N	4	1	1	1
Native	*Belgium*	Brussels	04°25' E	50°51' N	5	2	2	1
		Spa	05°50' E	50°29' N	4	2	2	1
	Denmark	Sundstrup	09°18' E	56°36' N	5	2	2	1
	England	Deal	01°24' W	51°13' N	4	2	2	1
	Finland	Kirkkonummi	24°31' E	60°09' N	5	1	1	1
	France	Mt. St. Michel	01°32' W	48°37' N	4	2	2	1
	Germany	Holzlarchen	11°43' E	47°53' N	4	2	2	1
		Lubeck	10°42' E	54°05' N	3	2	2	1
	Hungary	Csokvaomany	20°22' E	48°10' N	4	2	2	1
	Ireland	Near Caherdaniel	08°02' W	53°07' N	4	2	2	1
	Netherlands	Veluwe	06°00' E	52°19' N	5	2	2	1
		Wageningen	05°34' E	52°10' N	4	2	2	1
	Norway	Malvik	10°37' E	63°25' N	5	2	2	1
	Poland	Near Warsaw	19°25' E	51°52' N	5	2	2	1
	Scotland	Dundee	03°02' W	56°29' N	2	2	2	1
	Spain	Puerto de San Glorio	03°37' W	40°01' N	5	2	2	1
	Sweden	Lund	13°13' E	55°43' N	5	2	2	1
	Switerland	l'Himelette	07°00' E	47°07' N	5	2	2	1
		Rothenthurm	08°04' E	47°06' N	1	1	1	1



Supplementary Figure S1. Distribution map of native and invasive *J. vulgaris* populations from Europe (a), Australia and New Zealand (b) and USA (c) used in this study. For the native range, there are 19 populations from Europe. For the invasive range, there are 6 populations from Australia, 6 populations from New Zealand and 8 populations from USA.

Chapter 3

Parallel evolutionary changes in allocation to growth, defense and tolerance in invasive *Jacobaea vulgaris*

Tiantian Lin[1], Peter G.L. Klinkhamer[1], Thijs L. Pons[2],

Patrick P.J. Mulder[3], Klaas Vrieling[1]

[1] Institute of Biology, Section Plant Ecology and Phytochemistry, Leiden University, PO Box 9505, 2300 RA Leiden, The Netherlands

[2] Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, PO Box 800.84, 3508 TB Utrecht, The Netherlands

[3] RIKILT - Wageningen UR, PO Box 230, 6700 AE Wageningen, the Netherlands

Abstract

The Evolution of Increased Competitive Ability (EICA) hypothesis predicts that invasive plants invest less in defenses against herbivores and more in increased plant growth and competitive ability. As an extension, the Shifting Defense Hypothesis (SDH) predicts that invaders not only invest less in defense but in addition shift their defenses from more costly quantitative defenses to less costly qualitative defenses. In this study we examined plant growth, the underlying growth traits such as specific leaf area and photosynthesis, plant qualitative defense (pyrrolizidine alkaloids) and tolerance (regrowth-related traits such as carbohydrate storage and root inulin-structural root ratio) in native and invasive populations of *Jacobaea vulgaris* after 8 weeks of growth in a climate chamber. Our result showed that invasive *J. vulgaris* genotypes have been selected to evolve (1) better growth performance (2) higher pyrrolizidine alkaloids concentration, and (3) smaller investment in regrowth ability. All the studied traits measured in the invasive *Jacobaea vulgaris* populations from four geographically distinct regions changed consistently in the same direction. This indicates that parallel evolution took place in these regions, which differed significantly with respect to climatic conditions. The latter makes it likely that the observed evolutionary changes were caused by shifts in herbivore guilds.

Keywords

Biological invasions; Ragwort; Carbohydrate storage; Inulin; Regrowth; Pyrrolizidine alkaloids; Evolution of Increased Competitive Ability Hypothesis; Shifting Defense Hypothesis.

Introduction

Invasive plants provide an excellent opportunity for ecologists to study evolutionary changes by considering invasions as large-scale and long-term experiments in which major alterations in selective forces have occurred (Turner et al. 2014). One of the most commonly accepted theories assumes that the major change in selective forces on the allocation pattern of invasive plant species is the release from their specialist natural enemies (Enemy release hypothesis, ERH, (Williamson 1996; Keane and Crawley 2002; Mitchell and Power 2003). Since plant defenses can be costly (Koricheva 2002; Strauss et al. 2002) and many defensive traits are genetically controlled (Fritz and Simms 1992), such a shift in the herbivore composition towards a guild that is dominated by generalist herbivores is expected to exert altered selection on invasive plants that in turn leads to evolutionary changes towards decreased investment in anti-herbivore strategies. Such a decrease allows for an increased investment in growth during invasion (Colautti et al. 2004). As a result, invasive plants can outcompete local plant species as predicted by the Evolution of Increased Competitive Ability hypothesis (EICA) (Blossey and Nötzold 1995) and the Shifting Defense Hypothesis (SDH) (Müller-Schärer et al. 2004; Joshi and Vrieling 2005).

Although evolutionary changes towards higher growth rates as predicted by EICA have been observed in several invasive species (Pattison et al. 1998; DeWalt et al. 2004; Jakobs et al. 2004; Feng et al. 2009; van Kleunen et al. 2010), there is no strong evidence to prove that the absence of specialist herbivores is indeed the main responsible selective force as other biotic or abiotic factors cannot be ruled out as being important (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Felker-Quinn et al. 2013; Colomer-Ventura et al. 2015). Therefore in this study we compared evolutionary changes in multiple invasive regions that differ in climatological conditions. Parallel evolution in invasive plant from geographically and climatologically different ranges, would indicate that indeed changes in the herbivore guild are the most likely main selective force for the observed evolutionary changes. To our knowledge, this study is one of the first that examined plant growth, anti-herbivore defense and regrowth ability simultaneously in multiple invasive regions.

Higher growth rate is expected to be accompanied by a change in underlying factors such as increased photosynthesis, higher Specific Leaf Area (SLA, the ratio of total leaf area and leaf dry mass) and higher Leaf Mass Fraction (LMF, the ratio of leaf dry mass and total dry mass) (Poorter and Remkes 1990; Poorter 1999; Shipley 2006). In addition, Photosynthetic Nitrogen Use Efficiency (PNUE), the rate of photosynthetic capacity per unit nitrogen), has been reported to be positively associated with specific leaf area and plant growth (Poorter and Evans 1998). Several invasive plant species had higher growth than their native congeners and have been observed to evolve higher light-saturated photosynthetic rates (Pmax), SLA, LMF and PNUE (Durand and Goldstein 2001; McDowell 2002; Onoda et al. 2004; Nagel et al. 2005; Feng et al. 2009; Feng et al. 2011; Qing et al. 2012). On the other hand, Root Mass Fraction (RMF, the ratio of root dry mass and total dry mass = 1-LMF) is considered as a measure for root biomass allocation or partitioning in plants (Wang and Taub 2010). We therefore examined plant growth and the underlying growth traits SLA, LMF, RMF, Pmax and PNUE for the assessment of plant growth performance.

As predicted by the Shifting Defense Hypothesis, in the invasive range where specialist herbivores are largely absent, plants should increase cheap qualitative defenses, e.g. toxins such as glucosinolates, alkaloids or other groups of bioactive compounds that are effective even at low concentrations against local generalist herbivores. At the same time, plants should lower their costly quantitative defenses, which act as digestibility reducers such as tannins, that are only effective at relatively high concentrations, and thick leaves. As a result a net gain can be obtained by invasive plants for additional growth and increased competitive ability (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011). Studies related to plant anti-herbivore defense mostly focus on herbivore performance and so far few have measured changes in defense directly (Doorduin and Vrieling 2011).

Tolerance, the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores (Agrawal et al. 1999; Strauss and Agrawal 1999; Fornoni 2011), is regarded as a last resort against specialist herbivores which have broken through other defences. In terms of the Shifting Defense Hypothesis, it can be considered as costly quantitative defense since leaf tissue is lost through herbivory and reserves for tolerance cannot be used for growth (Bossdorf et al. 2004). Regrowth, the most common tolerance strategy, is a compensatory response to replace damaged tissue after herbivory. In order to cope with aboveground damage, plants store part of their resources in a safe place such as root to recover photosynthetic organs when removed using the stored material (Iwasa and Kubo 1997). Roots serve different functions for plants. Roots are used by plants for nutrient and water uptake, for storage of resources, and for the stability of the plant. A large RMF may be the result of high storage but can also be the consequence of low nutrient availability (van der Meijden et al. 2000). We suggest that it is crucial to study the root storage and structural components separately rather than associate the entire root with plant regrowth ability. Furthermore, high levels of regrowth ability are at the costs of higher growth rates since regrowth after defoliation requires high levels of stored resources in roots that can be used to produce new shoots (van der Meijden et al. 1988). In the introduced range, where the specialist herbivores are absent, we expect selection for a reduced allocation to regrowth and hence a lower storage of carbohydrates in the roots.

In this study we focused on the evolutionary changes in the allocation pattern between growth and anti-herbivore strategies of invasive plants using *Jacobaea vulgaris* (synonym *Senecio jacobaea*, Common ragwort) as study model. In the native areas *J. vulgaris* is under strong selection pressure by specialist herbivores. We compared invasive and native *J. vulgaris* populations and hypothesized that invasive populations in all invasive ranges (1) have increased growth and similar changes in the underlying growth traits, (2) have higher levels of qualitative defense and (3) have lower levels of regrowth and similar changes in the underlying traits. As qualitative defense we studied concentration and composition of pyrrolizidine alkaloids. We studied the regrowth capacity by examining carbohydrate storage in roots. In addition, all these traits were studied in four introduced populations from geographically and climatically distinct regions (Australia, New Zealand, West and Eastern coast of North America). Because only recently specialist herbivores were introduced in the invasive range as biological control agents, selection pressure of specialist herbivores has

been absent throughout the invasive range over 100 years, we expected parallel evolution to occur in invasive *J. vulgaris* populations in those four ranges.

Material and methods

Study species

Jacobaea vulgaris (synonym *Senecio jacobaea*, Common ragwort) is a monocarpic perennial plant that belongs to the family of the *Asteraceae*. It forms rosettes in the first year and flowers in the second year under favourable conditions. It is native to Eurasia and was introduced into parts of Australia (Harper and Wood 1957), New Zealand (Poole and Cairns 1940), Eastern coast of North America since the 1850s and Western coast of North America since 1900 (Harris et al. 1971). The ragwort populations of the west and east coast of North America are geographically isolated and thought to be independent introductions. Doorduin et al. (2010) found that the amount of genetic variation of native *J. vulgaris* populations does not differ from that of the different invasive ranges, suggesting that introductions from multiple source populations have occurred. Moreover, an assignment analysis indicated that populations from the Northern-west coast of Europe are the most likely source populations.

Jacobaea vulgaris is recorded to contain more than 37 different pyrrolizidine alkaloids (PAs), as constitutive, qualitative defences against herbivores (Witte et al. 1992; Cheng et al. 2011). Vrieling et al. (1993) found that 50%-100% of the phenotypic variance in the concentration and composition of PAs is under genetic control. Since PAs are toxic to horses and cattle, which can result in significant livestock losses due to alkaloid poisoning and decreased pasture yields, it received a pest status in the introduced range (Coombs et al. 1996). In a common garden experiment, Joshi and Vrieling (2005) found that invasive *J. vulgaris* had on average 90% more total amount of PAs (especially the jacobine type PA) and 37% higher reproductive biomass than genotypes from the native range. Furthermore, other studies found that PAs played an important role in plant resistance to several generalist herbivores (van Dam et al. 1995; Macel et al. 2005; Leiss et al. 2009). In the invasive ranges the herbivore guilds of *J. vulgaris* have been reported to be mainly dominated by local generalist herbivores (Poole and Cairns 1940; Stastny et al. 2005) while in the native range *J. vulgaris* is attacked by more than 70 herbivores, of which two main specialist herbivores, the cinnabar moth (*Tyria jacobaeae*) and the flea beetle (*Longitarsus jacobaeae*), cause most of the damage in Western Europe (van der Meijden et al. 2000). Both these specialist herbivores are adapted to PAs and sequester PAs (Aplin and Rothschild 1972; Zoelen and Meijden 1991; Dobler et al. 2000). *T. jacobaeae* uses PAs as oviposition and feeding stimulants (Macel and Vrieling 2003; Bernays et al. 2004; Cheng et al. 2013). In the past few decades, *T. jacobaeae* and *L. jacobaeae* were introduced as biological control agents into to the invasive range (Syrett 1983; McEvoy et al. 1991; McEvoy and Coombs 1999; McLaren et al. 2000). But so far no evolutionary adaptation of invasive *J. vulgaris* populations has been observed after the exposure to introduced *L. jacobaeae* (Rapo et al. 2010).

Plant material and growth conditions

Seeds were collected from 46 native populations in Europe and from 31 invasive populations in Australia, New Zealand, Western North America and Eastern North America (see Fig. 1 and Supplementary Table S1 for detailed population information). For each population seeds from three different (mother) plants were germinated in petri dishes with moistened filter paper. Two weeks after germination, from each mother plant 1 well-grown seedlings was potted in 0.5 L pots with 20% potting soil (Slingerland potgrond, Zoeterwoude, The Netherlands), 80% sandy soil from the dunes (collected from Meijendel, The Netherlands, 52º13'N, 4º34'E) and 0.75g Osmocote slow release fertilizer (Scott®, Scotts Miracle-Gro, Marysville, OH, USA; N:P:K:MgO 15:9:11:2.5). In a previous study the fresh weight of 600 seedlings from 20 native and 20 invasive populations were measured at the same age (two weeks after germination) and no significant differences were found between the origins (Lin et al. 2015b). In total the experiment contained (46 native populations + 31 invasive populations) * 3 = 231 plants. Plants were grown in the climate room for 9 weeks at 20°C, 70% humidity, 16 hours day light with a light intensity of 113 µmol $m^{-2}s^{-1}$PAR. Eight native plants that showed stunted growth were excluded from the measurements.

Measurements of underlying growth traits

Photosynthesis and leaf nitrogen measurement

After six weeks of growth in the climate chamber, the light saturated rate of photosynthesis per unit leaf area (Pmax) was measured on the middlemost leaf of each plant using a LICOR 6400 (Portable Photosynthesis analyser, LiCor Inc. Lincoln, NE, USA) at atmospheric CO2, (ca. 380 µmol mol^{-2} in the leaf chamber), growth temperature and 1500 µmol $m^{-2}s^{-1}$ PAR. Before the measurement leaves were pre-lighted for 5 minutes to saturate photosynthesis. Each leaf was measured three times consecutively. Before statistical analysis the average of the three measurements was taken. The part of the leaf used for the photosynthesis measurement was dried in oven at 50°C for 3 days. After that the nitrogen (N) concentration were measured using a CHN analyzer (Carlo Erba, Milan, Italy) and the nitrogen content per unit leaf area were calculated based on the same leaf. In a previous study the nitrate concentration was analyzed in the leaves of *J. vulgaris* and only trace amount of nitrate were found, which indicates *J. vulgaris* is not a nitrate accumulator (data not shown).

Growth measurements

After 9 weeks, all 223 well-grown plants were harvested, the total leaf area of each plant was measured by a portable leaf area meter (LI-3100, LI-COR, Inc., Lincoln, NE, USA). Dry mass of shoots and roots were measured separately after oven drying at 60°C for 3 days. Leaf and root materials for later analysis were also accounted in the dry mass and the leaf area. SLA (cm^2 g^{-1}) was calculated as the ratio between total leaf area and shoot dry mass. LMF (g g^{-1}) was calculated as the ratio between shoot dry mass and plant total mass. RMF (g g^{-1}) was calculated as the ratio between root dry mass and plant total mass. PNUE (µmol CO_2 $g^{-1}s^{-1}$) was calculated as the ratio of Pmax to leaf N per leaf area.

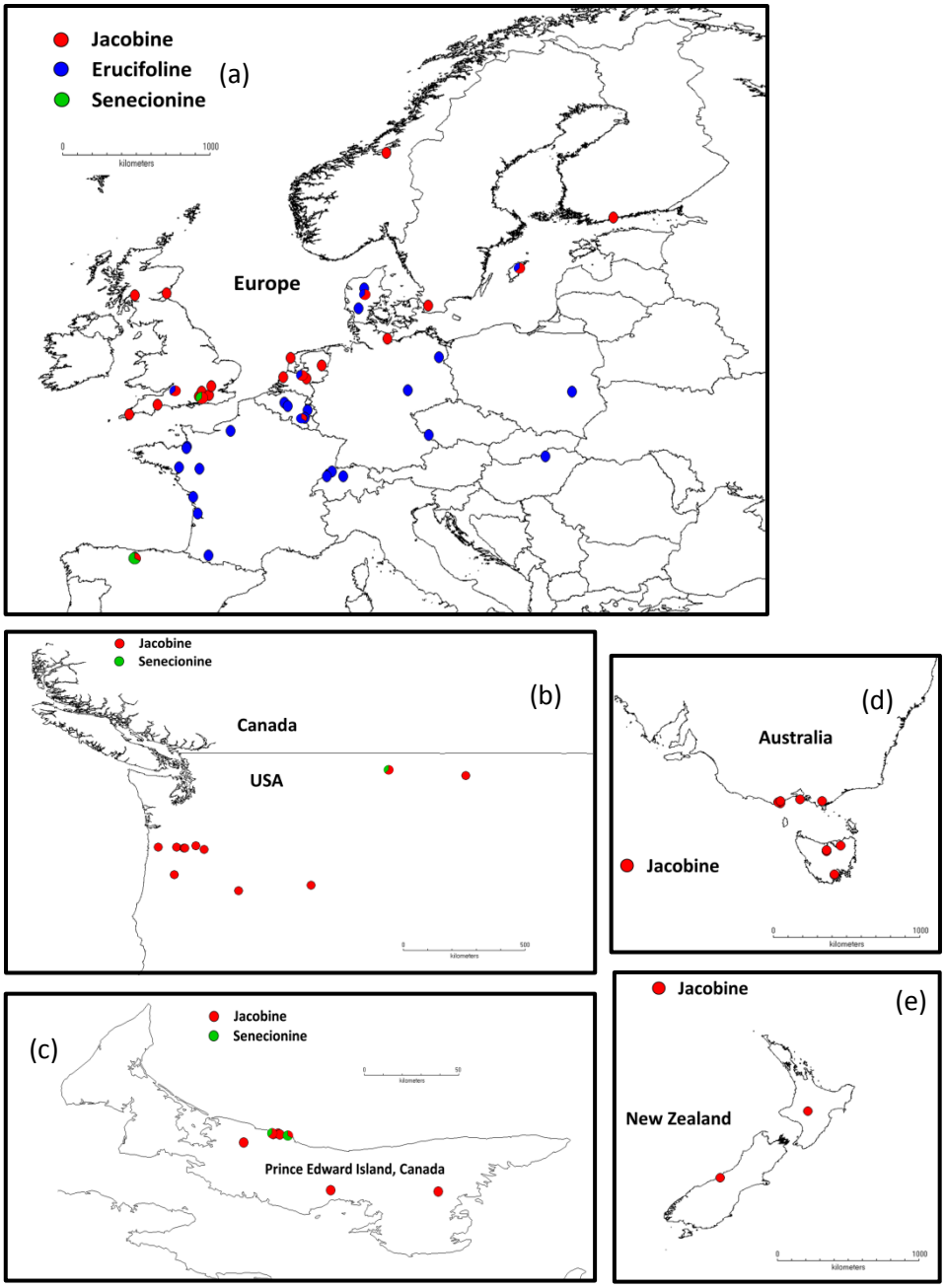


Fig 1. Distribution map of native and invasive *Jacobaea vulgaris* populations used in this study from Europe (a, n=46 populations), Western North America (b, n=13 populations), Eastern North America (c, n=6 populations), Australia (d, n=10 populations) and New Zealand (e, n=2 populations), Different colors indicated different chemotypes in each population and the proportion of different colors in one population indicated the number of genotypes from corresponding chemotypes (1 or 2 out of 3 motherplants).

Qualitative defense

Pyrrolizidine alkaloids extraction and analysis

Two middle leaves of each plant were freeze-dried and approximately 10 mg of powdered leaf material was extracted with 1 ml 2% formic acid containing 1 µg/ml heliotrine as an internal PA standard. The plant extract solution was shaken for 30 min. Solid plant material was removed by centrifugation at 720×g for 10 min and the supernatant was filtered through an 0.2-µm nylon membrane (Acrodisc 13-mm syringe filter, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot of the filtered solution (25 µl) was diluted with 10 mM ammonia (975 µl) and 10 µl was injected into the LC-MS⁄MS system (Acquity UPLC system coupled to a Quattro Premier tandem mass spectrometer (Waters, Milford, MA, USA). PAs were separated on a Waters UPLC BEH C_{18} (150 x 2.1 mm, 1.7 µm) analytical column using a 12-min acetonitrile/water (pH 12) gradient. The analytical method included 44 PAs, of which 20 were available as reference standards. Analysis by LC-MS/MS was conducted according to Cheng et al. (2011) and the instrumental settings are available as supporting information. Data processing was conducted with Masslynx 4.1 software (Waters Corporation, Milford, MA, USA).

PAs can occur in plants in two forms: tertiary amine (free base) and N-oxide (Areshkina 1950; Hartmann and Toppel 1987). According to the structural characteristics and biosynthetic pathways PAs were classified into four groups: senecionine (Sn)-, jacobine (Jb)-, erucifoline (Er)- and otosenine (Ot)-like PAs (Pelser et al. 2005; Cheng et al. 2011). Based on relative presence or absence of the Jb- and Er-like PAs, *Jacobaea vulgaris* individuals were classified as Sn, Jb, or Er chemotypes (Witte et al. 1992; Macel et al. 2004). We considered plants with mainly Jb-like PAs and no or little Er-like PAs as Jb chemotypes, plants with mainly Er-like PAs and no or little Jb-like PAs as Er chemotypes, and plants that had little Er- and Jb-like PAs but larger amounts of Sn-like PAs as Sn chemotypes.

Regrowth

Root inulin extraction (Carbohydrate storage)

Inulin is the natural carbohydrate storage source found in roots of the Asteraceae (Tertuliano and Figueiredo-Ribeiro 1993). Root inulin concentration was measured as the difference between the total sugar content after hydrolysis and the free sugar content before hydrolysis. 0.1g ground dried root material was incubated with 4 mL distilled water at 80°C for 1 hour. After centrifuging the free sugar content was measured by adding 2mL 3,5-dinitrosalicylic acid (DNS) to 1 ml of the supernatant and measuring the absorbance with a spectrophotometer at 540nm. The concentration was calculated using a calibration line made with D(-) fructose according to Miller (1959). The total sugar content of the remaining 1mL supernatant was hydrolysed by 200µl inulinase (Novozym®960, Sigma-Aldrich) for 1 hour at 60 °C and measured by the same DNS method. Root total inulin content was calculated as the root inulin concentration multiplied by total root dry mass.

Since root biomass consists of both storage and structural tissues, we made a distinction between storage root dry mass (= root inulin content) and the structural root dry mass (= total root dry mass – root inulin content). Furthermore, the root inulin-structural root ratio and shoot-structural root ratio were calculated as indicators for regrowth ability and growth ability, respectively.

Climatic conditions

To examine the difference in the local climate among the five geographic regions (Europe, Australia, New Zealand, Western North America, Eastern North America), 19 bioclimatic variables of the current conditions (ca 1950–2000) were downloaded from the WorldClim dataset (http://www.worldclim.org/current) in 5 arc-minutes resolution for each sampled population.

A partial least square-discrimination analysis (PLS-DA) was performed with the SIMCA-P software (v.11.0, Umetrics, Umeå, Sweden) for classifying all sampled populations based on the 19 bioclimatic variables. The scaling method for PLS-DA was unit-variance and the model was validated by using the permutation method through 20 iterations.

Statistical analysis

As the main interest of this study was to find differences between invasive and native populations, statistical analyses were performed with nested ANOVAs, with origin (native-invasive) as fixed factor, region nested with origin and population nested within region as random factors. Normality of the residuals were checked with a Shapiro-Wilk test. A log transformation was conducted for SLA, shoot- structural root ratio, root inulin-structural root ratio and all PA related traits to obtain a normal distribution of the residuals. Furthermore, in order to exam the differences among the invasive and native regions (Australia, New Zealand, Western North America, Eastern North America and Europe), a post hoc LSD test was used for each trait which differed significantly between invasive and native populations. All analyses were carried out using SPSS 18.0 (SPSS: An IBM Company, Wacker Drive, Chicago, USA).

Results

Climatic conditions

A PLS-DA plot based on the 19 bioclimatic variables shows that the sampled populations of the four invasive regions are clearly separated (Fig. 2).

Growth and underlying growth traits

Invasive *J. vulgaris* populations had 27% heavier shoots, 15% smaller roots and 12% larger total dry mass than their native congeners (Table 1). In addition, invasive populations had 5% and 12% higher SLA and LMF respectively but a 23% smaller RMF than native populations. When the structural root (total root mass minus inulin mass) no difference was found in

structural root dry mass between invasive and native populations but invasive populations had a 15% higher structural shoot-root ratio than native ones. On average, invasive *J. vulgaris* genotypes had a 7.7% and 10.8% higher Pmax and PNUE than native ones though leaf nitrogen content per unit leaf area was not significantly different from native populations.

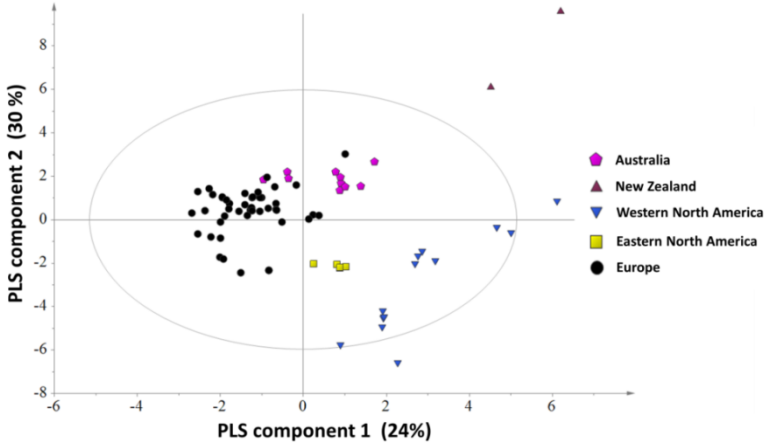


Fig. 2 Partial least square-discrimination analysis (PLS-DA) plots for classifying the five geographic regions based on the 19 bioclimatic variables from the collected site of each sampled population (N=46 for Europe, N= 10 for Australia, N=2 for New Zealand, N=13 for Western North America and N=6 for Eastern North America). The two outliners from New Zealand were mainly due to the high annual precipitation.

Qualitative defense

On average, the invasive genotypes had a 43% higher total leaf PA concentration than plants from the native genotypes. The increase in total leaf PA concentration in the invasive area was caused by an increase of 123% in total tertiary amine concentration while no significant difference was found for the total N-oxide PA concentration (Table 2). Furthermore, invasive populations had significantly higher Sn- and Jb- like PAs but less Er- and Ot-like PAs than native populations.

In the native range, two major chemotypes were found. The Jb chemotype is mostly distributed along the coasts of Northwest Europe (Fig. 1a, red dots). In contrast, the Er chemotype is predominant along the Southwest coast of Europe and more inland Europe (Fig. 1a, blue dots). Interestingly, the two genotypes from Spain and one genotype from UK were classified as Sn chemotypes (Fig. 1a, green dots). In the invasive range, 96% of the genotypes were found to be Jb chemotypes. Four percent of the invasive genotypes were Sn chemotypes and were found only in North America (Fig. 1b, 1c and 1d, green dots). When comparing invasive and native jacobine chemotype plants only, no significant differences were found in the levels of total PAs, N-oxides PAs and jacobine-like PAs (Table 3). However, the invasive Jb chemotypes contained significantly higher PA tertiary amines concentration than native Jb chemotypes. Moreover, the invasive genotypes contained significantly more Sn-like PAs and less Er- and Ot-like PAs compared to native ones. This indicates that the

composition and concentration of PAs in invasive Jb chemotypes have been changed and that these changes occurred in the same direction for all four invasive regions.

Table 1. Growth differences and underlying growth traits between invasive and native *J. vulgaris* populations. P values are from a nested ANOVA, with origin (native versus invasive) as a fixed factor, region nested within origin and population nested within region as random factors.

Variables	***Invasive***	***Native***	***P (origins)***	***P (regions)***	***P (populations)***
Total dry mass (g)	3.73±0.14	3.33±0.11	0.041	NS	0.007
Shoot dry mass (g)	2.72±0.10	2.14±0.07	<0.001	NS	0.002
Root dry mass (g)	1.01±0.05	1.19±0.05	0.031	NS	0.003
Specific leaf area ($cm^2\ g^{-1}$)	238.09±2.14	228.3±2.48	0.044	NS	0.013
Leaf mass fraction ($g\ g^{-1}$)	0.728±0.005	0.649±0.006	<0.001	NS	0.013
Root mass fraction ($g\ g^{-1}$)	0.272±0.005	0.351±0.006	<0.001	NS	0.013
Structural root dry mass (g)	0.618±0.028	0.594±0.027	NS	NS	NS
Structural shoot-root ratio ($g\ g^{-1}$)	4.71±0.16	4.11±0.14	0.012	NS	NS
Leaf N content per unit area ($g\ m^{-2}$)	1.25±0.02	1.30±0.02	NS	NS	NS
Pmax (μmol $CO_2\ m^{-2}s^{-1}$)	23.13±0.43	21.48±0.38	0.005	NS	NS
PNUE (μmol $CO_2\ g^{-1}s^{-1}$)	18.86±0.44	17.02±0.41	0.011	NS	0.001

Values are mean values ± SE, df=1, 3. *P* (origins): significance level of nested ANOVA between invasive and native origins. P (regions): significance level of nested ANOVA among regions. *P* (populations): significance level of nested ANOVA among populations. NS= not significant. Structural root dry mass is the root dry mass minus root inulin content.

Table 2. The differences of leaf qualitative defenses (Pyrrolizidine alkaloids) of all chemotypes between invasive and native *J. vulgaris* populations. Variables are all showed as concentration ($\mu g\ g^{-1}$ DM). Averages were tested with a nested ANOVA, with origin (native versus invasive) as a fixed factor, region nested within origin and population nested within region as random factors.

Variable	***Invasive***	***Native***	***P (origins)***	***P (regions)***	***P (populations)***
Total PA	4145±153	2893±163	0.001	NS	<0.001
Total PA (Tertiary amines)	1955±90	877±98	<0.001	NS	<0.001
Total PA (N-oxides)	2190±114	2016±105	NS	NS	0.002
Senecionine-like PAs	1252±86	782±64	0.004	NS	<0.001
Jacobine-like PAs	2877±131	1461±1722	<0.001	NS	<0.001
Erucifoline-like PAs	9.5±7.6	639±51	<0.001	NS	<0.001
Otosenine-like PAs	0.01±0.01	1.7±0.53	0.35	NS	NS

Values are mean values ± SE, df=1, 3.*P* (origins): significance level of nested ANOVA between invasive and native origins. P (regions): significance level of nested ANOVA among regions. *P* (populations): significance level of nested ANOVA among populations. NS= not significant.

Table 3. The leaf qualitative defenses (Pyrrolizidine alkaloids) of jacobine chemotypes only from invasive and native *J. vulgaris*. Variables are all showed as concentration (μg g^{-1} DM). Averages were tested with a nested ANOVA, with origin (native versus invasive) as a fixed factor, region nested within origin and population nested within region as random factors.

Variable	***Invasive***	***Native***	***P (origins)***	***P (regions)***	***P (populations)***
Total PA	4188 ± 155	4351 ± 191	NS	NS	0.022
Total PA (Tertiary amines)	2039 ± 83	1870 ± 126	0.029	NS	0.001
Total PA (N-oxides)	2149 ± 114	2480 ± 154	NS (0.071)	NS	0.018
Senecionine-like PAs	1176 ± 376	833 ± 84	0.007	NS	<0.001
Jacobine-like PAs	3004 ± 120	3143 ± 147	NS	NS	0.005
Erucifoline-like PAs	0.64 ± 0.09	356 ± 53	<0.001	NS	<0.001
Otosenine-like PAs	0.01 ± 0.01	1.48 ± 0.59	0.024	NS	0.002

Values are mean values ± SE, df=1, 3. *P* (origins): significance level of nested ANOVA between invasive and native origins. P (regions): significance level of nested ANOVA among regions. *P* (populations): significance level of nested ANOVA among populations. NS= not significant.

Regrowth

Invasive *J. vulgaris* populations had a 23% and 34% lower root inulin concentration and total root inulin content, respectively, compared to the native population, resulting in a 37% lower root inulin-structural root ratio in invasive populations (Table 4). No difference was found in the free sugar concentration between the invasive and native populations.

Table 4. The differences of regrowth related traits between invasive and native *J. vulgaris* populations. Averages were tested with a nested ANOVA, with origin (native versus invasive) as a fixed factor, region nested within origin and population nested within region as random factors.

Variables	***Invasive***	***Native***	***P (origins)***	***P (regions)***	***P (populations)***
Inulin concentration in root (g g^{-1} DM)	0.376±0.018	0.485±0.017	<0.001	NS	0.045
Total inulin content in root (g)	0.397±0.025	0.597±0.028	<0.001	NS	0.001
Free sugar concentration in root (g g^{-1} DM)	0.116±0.004	0.126±0.005	NS	NS	0.001
Root inulin-structural root ratio (g g^{-1})	0.723±0.059	1.156±0.065	0.012	NS	NS

Values are mean values ± SE, df=1, 3. *P* (origins): significance level of nested ANOVA between invasive and native origins. P (regions): significance level of nested ANOVA among regions. *P* (populations): significance level of nested ANOVA among populations. NS= not significant. Root inulin-structural root ratio is calculated as the root inulin content divided by the subtract between total root dry mass and root inulin content.

Parallel evolution

In general, the results showed that of all measured traits related to growth, chemical defense and regrowth, that were significantly different between invasive and native *J. vulgaris* populations, the invasive populations from the four geographically distinct regions changed in the same direction (Fig. 3). They were all significantly differed from native populations except for the 2 populations from New Zealand that showed no significant differences in 4 traits but still deviated in the predicted direction.

Discussion

Parallel evolution among invasive regions

In this study we found that all traits measured (growth ability, qualitative defense and regrowth ability) that significantly differed between invasive and native *J. vulgaris* populations, the invasive populations from the four geographically distinct regions changed in the same direction and all differed significantly from native populations (Fig. 3). The only exception was the populations from New Zealand which for some traits (shoot dry weight, total dry weight, specific leaf area and shoot to structural root ratio) showed no difference compared with the native populations. It should be remarked that the sample size for this particular region is quite low (only two populations were available), therefore the variance among populations was quite big which easily might render non-significant results. However, all traits deviated in the predicted direction even in the non-significant cases. Our results strongly suggest that parallel evolution has occurred in the invasive *J. vulgaris* populations from the four geographically distinct regions. It is worth to point out that there are alternative explanations e.g. bridgehead or anthropogenically induced adaptation to invasive regions (Lombaert et al. 2010; Hufbauer et al. 2012). However, genetic analyses showed that multiple introductions have occurred in the invasive ranges (Doorduin *et al.* 2010), suggesting that the changes in the traits can be best explained by parallel evolution in the invasive *J. vulgaris* populations from the four geographically distinct regions. Such parallel evolution is more likely due to the absence of selection pressures from specialist herbivores than to adaptation to local abiotic factors since the major abiotic factor, the local climatological conditions, are significantly different among the four invasive regions.

So far the Evolution of Increased Competitive Ability hypothesis has been tested by numerous studies and several invasive plant species have been observed to have increased growth performance with decreased herbivore resistance (Bossdorf et al. 2005; Hinz and Schwarzlaender 2009; Felker-Quinn et al. 2013). However, evidences collected from those studies are still insufficient to support the absence of specialist herbivore is the main selective force responsible for such post-invasion evolutionary changes. And besides the release from natural enemies, adaptation to local abiotic factors such as climate conditions could also play a potential role on the selection of invasive plants (Bradley et al. 2009; Colomer-Ventura et al. 2015). Thus in order to largely rule out other abiotic factors, it is essential to setup a study design where multiple invasive regions can be compared that are differ in climatological conditions.

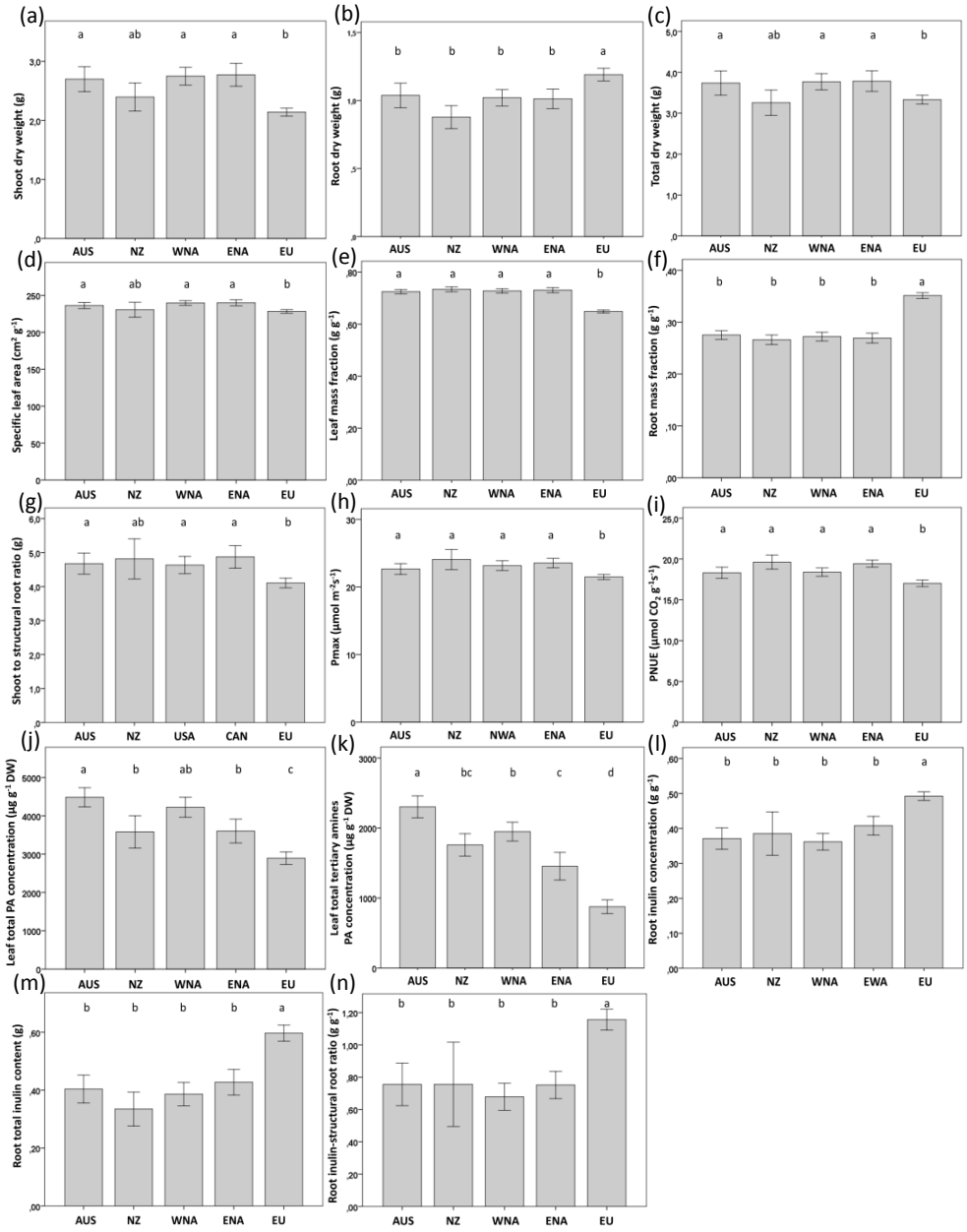


Fig. 3. The differences among regions of measured traits that are significantly differed between invasive and native *Jacobaea vulgaris* populations: shoot dry mass (a), root dry mass (b), total dry mass (c), specific leaf area (d), leaf mass fraction (e), root mass fraction (f), shoot- structural root ratio (g), Pmax (h), PNUE (i), leaf total PA concentration (j), leaf total tertiary amines PA concentration (k), root inulin concentration (l), root total inulin content (m) and root inulin-structural root ratio (n) among invasive populations from four geographic regions (Australia, New Zealand, Western North America and Eastern North America) and native populations from Europe. Values are means ± SE. Different letters indicate significant differences among treatments at $p<0.05$ according to a post hoc LSD test (ANOVA).

Growth

The major prediction of the Evolution of Increased Competitive Ability hypothesis is that the absence of herbivory by specialists leads to an evolutionary shift in energy allocation from defense to growth. In this study we found strong evidence that the invasive *J. vulgaris* populations have better growth performance compared to the native populations, based on growth and underlying traits (SLA, LMF, Pmax and PNUE). Furthermore, invasive populations allocated less inulin to the roots than the native populations and had a higher shoot-root ratio which is positively correlated with plant growth ability (Gedroc et al. 1996; Andrews et al. 1999). Indeed we found that invasive populations had a significantly larger total dry mass than the native ones. The larger vegetative biomass was shown by (Joshi and Vrieling 2005) to result also in a 37% higher in floresence mass for invasive *J. vulgaris* compared to native ragworts. As *J. vulgaris* is a monocarpic plant a higher reproductive mass directly translates into an increased fitness. All invasive populations had higher underlying growth traits like SLA and LMF that are positively correlated with relative growth rate (Poorter and Remkes 1990; Shipley 2006). In addition, as expected, invasive *J. vulgaris* had higher Pmax and their leaf nitrogen content was not differed from native populations. It contributes to an increased PNUE which indicates that invasive *J. vulgaris* could allocate a higher fraction of leaf nitrogen to photosynthesis and a lower fraction to light-harvesting components (Poorter and Evans 1998; Schieving and Poorter 1999; Feng et al. 2008; Feng and Fu 2008). Feng *et al.* (2008) found that invasive *Ageratina adenophora* had evolved an increased nitrogen allocation to photosynthesis and a reduced allocation to cell walls, suggesting a trade-off between the two traits. In an independent study we found that invasive *J. vulgaris* had a lower amount of cell wall proteins per unit leaf area than native genotypes (Lin et al. 2015a).

Qualitative defense

We found invasive *J. vulgaris* populations had on average higher total PA and tertiary amines PA concentrations than native populations. This is likely due to the fact that 96% of the invasive genotypes were Jb chemotype while 52% of the native genotypes were Er chemotype. In *J. vulgaris* the Jb chemotype contains a higher amount of tertiary amines than Er and Sn chemotypes because Jb-like PAs are present as tertiary amines in a higher percentage than the other PA types (Macel et al. 2004). Compare to the native Jb chemotype, the invasive Jb chemotype contained relatively more Sn-like PAs and only trace amounts of Er-like PAs. As a result the total tertiary amine concentration is significantly higher in the invasive populations, although no significant difference in total PA concentration was found between invasive and native Jb chemotype. Since tertiary amines have been found to be more deterrent to insects than the N-oxide PAs (Macel et al. 2005), the PA composition of invasive populations are potentially more toxic than native populations. Our findings are in line with Joshi & Vrieling (2005) who also found no Er chemotype presents in the invasive ranges. They suggested that the Er chemotype has either not been introduced to the invasive areas or has been selected against in the new environment. Furthermore, herbivory has been reported to affect PA composition in *J. vulgaris* (Hol et al. 2004) and generalist herbivores could play a role in the evolution and maintenance of the diversity of PAs (Macel et al. 2005). Therefore we argue that the changes in the herbivore guild towards generalists-dominant in the introduced areas could

have led to evolutionary selection on the PA composition in invasive *J. vulgaris*. In contrast, studies also found that the specialist herbivore *T. jacobaeae* is more sensitive to tertiary amines PA and the relative effects of individual PAs differed between herbivore species (Macel et al. 2005; Cheng et al. 2013; Wei et al. 2015). The difference in the PA composition between native and invasive *J. vulgaris* populations might also explain our previous finding that why the outcome of the competition is strongly depend on the type or level of herbivory (Lin et al. 2015b).

Regrowth

We found that invasive *J. vulgaris* populations had significantly less carbohydrate storage (inulin) in the root compared to native ones but not differ in structural root mass (Table 4). It indicates the different root mass we found between invasive and native *J. vulgaris* is due to their different investment in the root carbohydrate storage and invasive populations had allocated much less storage into the roots. Since the amount of root inulin storage at the moment of defoliation was found to be positive correlated with plant regrowth size one month after defoliation (chapter 4), it strongly suggests a better regrowth ability in the native populations. This is consistent with the finding that native *J. vulgaris* genotypes had better regrowth after complete defoliation by clipping in a competition-free condition in a common garden experiment (Joshi and Vrieling 2005) or after herbivory by either *Mamestra brassicae* or *T. jacobaeae* under intra-specific competition (Lin et al. 2015b). Most likely selection pressure by the specialist *T. jacobaeae* in the native range plays an important role. The foliar-feeding larvae of this specialist herbivore regularly defoliate all the aboveground parts of *J. vulgaris* in the native range while this is not the case for the herbivore guild that is dominated by generalist herbivores in the invasive range. The univoltine cinnabar moth could break through all plant defenses and that ragwort uses regrowth as a strategy to overcome defoliation by the cinnabar moth (Dempster 1971; van der Meijden et al. 1988). In contrast, it could be argued that the higher shoot-structural root ratio in the invasive *J. vulgaris* populations represents a redistribution of resources from root storage to growth of aboveground parts, and thus contributing to better growth performance.

In conclusion, we found invasive *J. vulgaris* populations have been selected to evolve better growth ability and higher qualitative defense but decreased regrowth ability compare to native populations. These results support the EICA and SDH hypotheses that due to the absence of adapted specialist herbivores, a net gain can be obtained for increasing growth by the invasive plants through shifting their defenses from more costly quantitative defense (tolerance) to less costly qualitative defense (chemical defense). Additionally, all the traits measured in the invasive *J. vulgaris* genotypes from four geographically distinct regions changed consistently in the same predicted direction. It can be explained by a parallel evolution in response to the absence of specialist herbivores after invasion since the climatological conditions were significantly different among these invasive regions.

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Supplementary Table S1. Origin of seeds from 46 native and 31 invasive populations of *J. vulgaris* used in this study. For each population three genotypes from different mother plants were used.

Origin	***Country***	***Location***	***Longitude***	***Latitude***
Invasive	Western North America	Cooper Mountain, Oregon	122°53' W	45°27' N
		Indian Creek, Oregon	117°49' W	44°01' N
		Sunrise Hill, Montana	115°00' W	48°15'N
		West Crestmont, Oregon	121°51' W	45°22' N
		Island Lake, Oregon	122°37' W	45°25' N
		Larch Slope, Oregon	122°10' W	45°30' N
		Conrad Spur, Montana	111°58' W	48°09' N
		No Bear Road, Oregon	120°33' W	43°48' N
		Island Lake Road, Montana	114°59' W	48°12'N
		Little Wolf, Montana	115°00' W	48°21' N
		Kootenai National Forest, Montana	114°53' W	48°17' N
		Salem, Oregon	122°95' W	44°87' N
		Cochran Creek, Oregon	122°58' W	44°25' N
	Eastern North America	Cardigan, Prince Edward Island	63°37' W	46°13' N
		Charlottetown, Prince Edward Island	63°07' W	46°14' N
		Green Cables, Prince Edward Island	63°22' W	46°29' N
		Clinton, Prince Edward Island	63°32' W	46°26' N
		Cavendish Beach, Prince Edward Island	63°24' W	46°29' N
		Marco Polo Campground, Prince Edward Island	63°22' W	46°29' N
	Australia	Franklin, Tasmania	147°01' E	43°05' S
		Dairy Plains 1, Tasmania	146°31' E	41°38' S
		Dairy Plains 2, Tasmania	146°32' E	41°34' S
		Wild Dog Road, Victoria	143°40' E	38°41' S
		Beech Forest, Victoria	143°33' E	38°38' S
		Barramunga, Victoria	143°41' E	38°34' S
		Cape Schanck, Victoria	144°54' E	38°27' S
		Turton's Creek, Victoria	146°15' E	38°33' S
		Targa, Tasmania	147°23' E	41°18' S
		Franklin, Tasmania	147°01' E	43°04' S
	New Zealand	Fox Glacier, South island	170°01' E	43°27' S
		Tongariro National Park, North island	175°34' E	39°14' S
Native	Belgium	Bertogne1	05°40' E	50°05' N
		Bertogne2	05°40' E	50°05' N
		Spa	05°50' E	50°29' N
		Brussels	04°25' E	50°51' N
		Louvain-la-Neuve	04°37' E	50°40' N

Denmark	Sundstrup	09°18' E	56°36' N
	Hovborg	08°56' E	55°36' N
	Engesvang	09°21' E	56°10' N
Finland	Masala, Kirkkonummi	24°31' E	60°09' N
	Inkoo, Kirkkonummi	24°00' E	60°02' N
France	Mont Saint Michel, Normandie	01°32' W	48°37' N
	Rouen, Normandie	01°05' W	49°26' N
	Pontorson, Normandie	01°36' W	48°33' N
	Les Essarts, Pays-de-la-Loire	01°10' W	46°07' N
	Lesparre-Medoc, Aquitaine	00°54' W	45°17' N
	Brillac, Bretagne	02°48' W	47°32' N
	Lamotte-Beuvron	02°01' W	47°36' N
	Lourdes, Midi-Pyrénées	00°13' W	43°09' N
Germany	Halle, Sachsen	11°58' E	51°29' N
	Lubeck, Schleswig-Holstein	10°42' E	54°05' N
	Nassenheide, Brandenburg	13°14' E	49°14' N
	Pfingstberg, Brandenburg	13°52' E	53°08' N
Hungary	Csokvaomány	20°22' E	48°10' N
	Lénárddaróc	20°22' E	48°08' N
Netherlands	Meijendel	04°20' E	52°07' N
	Wageningen	05°34' E	52°10' N
	Mossel	05°45' E	52°03' N
	Gees	06°41' E	52°44' N
	Texel	04°48' E	53°05' N
Norway	Malvik	10°37' E	63°25' N
Poland	Pulawy	21°59' E	51°24' N
Spain	Covadonga	04°45' W	43°04' N
Sweden	Lund	13°13' E	55°43' N
	St Olofsholm, Gotland	18°54' E	57°43' N
Switzerland	Mettembert	07°19' E	47°23' N
	Saint-Imier	07°00' E	47°09' N
UK	Falls of Leny, Scotland	04°16' W	56°15' N
	Bentley, Hampshire	00°51' W	51°10' N
	Alice Holt Forest, Hampshire	00°50' W	51°10' N
	Silwood Park, Berkshire	00°38' W	51°24' N
	Saint Andrews, Scotland	02°47' W	56°20' N
	Sevenoaks Weald, Kent	00°12' W	51°14' N
	Marsh Green, Devon	03°21' W	50°44' N
	Threemilestone, Cornwall	05°05' W	50°16' N

Native	UK	Enfield, Greater London	00°03' W	51°40' N
		Corston, Bath	02°26' W	51°23' N

Chapter 4

Parallel evolution in invasive *Jacobaea vulgaris*:

The effect of carbohydrate storage on growth and regrowth

Tiantian Lin, Peter G.L. Klinkhamer, Klaas Vrieling

Institute of Biology

Section Plant Ecology and Phytochemistry

Leiden University, PO Box 9505, 2300 RA Leiden

The Netherlands

Abstract

Invasive plants are expected to have evolved decreased regrowth ability and increased growth due to the absence of specialist herbivores in the introduced ranges. We compared growth and regrowth ability of invasive and native *Jacobaea vulgaris* in response to simulated shoot defoliation. The results showed that while native populations regrew better after defoliation, the non-defoliated invasive populations had better growth. All the studied traits of invasive populations from three geographically and climatologically distinct regions differed from native populations and changed in the same direction. This implies that it is most likely that the shift in herbivore guilds were causal to the evolutionary changes rather than other environmental factors. Our data further suggests that regrowth ability is positively associated with root carbohydrate storage while negatively associated with structural root mass, so that is it essential to study root storage and root size separately in order to investigate plant regrowth.

Keywords

Biological invasions; Inulin; Evolution of Increased Competitive Ability hypothesis; Shifting defense hypothesis; Ragwort; Herbivore resistance; Tolerance; Plant defense.

Introduction

Although the introduction of invasive plant species in a given area causes economic and ecological problems, it also provides an ideal opportunity for ecologists to study evolutionary changes, if considered as large-scale and long-term experiments where major alterations in selective forces have occurred. The most striking change after invasion is that of the herbivore guild. Though still under herbivore pressure by generalist herbivores and occasional specialist herbivores of congeneric plant species, the invasive species are freed from their native specialist herbivores (Frick 1972; Castells et al. 2013). Since plant defense can be costly (Koricheva 2002; Strauss et al. 2002) and many defense traits are genetically controlled (Fritz and Simms 1992), a shift in the herbivore composition towards a guild that is dominated by generalist herbivores can be expected to exert an altered selection on the invasive plants. At the same time, this should in turn, result in evolutionary changes that lead to a decrease in the anti-herbivore strategies used to deal with the "old" specialist herbivores that have ceased to exist after invasion. In this case, a net resource used for defense could be reallocated to plant growth thus increasing their competitive ability over the local plant species and allowing a higher seed reproduction as proposed in the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Nötzold 1995; Lin et al. 2015).

However, evidence collected so far to support the idea that changes in the herbivore guild are the selective force for changes in allocation patterns is largely circumstantial (Willis et al. 1999; Colautti et al. 2004; Colomer-Ventura et al. 2015). In order to rule out other biotic and/or abiotic factors that could potentially have an effect on this, we set out to study a system in which multiple invasive regions that differed in climatological conditions were compared. If changes in the herbivore guild were the main selective force for growth and regrowth, parallel evolutionary changes could be expected in each of the different geographical and climatological invasive ranges.

Among plant strategies developed to cope with herbivore pressure, an alternative to herbivore deterrence is the development of tolerance to damage by reducing the negative fitness effects of the herbivores (van der Meijden et al. 1988). Tolerance has been defined as the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores (Strauss and Agrawal 1999). Herbivore pressure plays a major role in the selection of plants to evolve tolerance. When some specialist herbivores have broken through all plant defenses, tolerance is the only strategy left for plants to overcome defoliation after herbivory (Dempster 1971; van der Meijden et al. 1988). A variety of plants suffer from high levels of mainly specialist herbivory that result in frequent defoliation during their life time (Briske and Richards 1995). The level of tolerance they exhibit may vary among sites though, according to the history of grazing damage (Fornoni 2011). For example, plant species with a long history of grazing history have a higher level of tolerance than those with a shorter history (Lennartsson et al. 1997). Therefore in the native ranges where specialist herbivores are present, the level of tolerance of native genotypes is expected to be higher than in the invasive area where specialist herbivores are absent.

However, tolerating damage by herbivores incurs a fitness cost. Plant tolerance is considered to be costly because tissue is lost through herbivory and perhaps even more importantly, in the case of fast-growing plants, the reserves allocated to tolerance cannot be used for growth (Bossdorf et al. 2004). Therefore, in absence of herbivores, plant genotypes that have high levels of tolerance may have a poorer growth performance than genotypes with lower tolerance. Although tolerance plays a significant role in the way plants cope with herbivore attack (Strauss and Agrawal 1999), its evolutionary consequences have rarely been studied within the framework of the Evolution of Increased Competitive Ability (EICA) hypothesis. Invasive species that have been devoid of their specialist herbivores for many generations could be an ideal system to study the selective pressure on tolerance. In this study we tested the hypothesis that in this situation, invasive plants have evolved towards a decreased investment in tolerance and an increased investment in growth.

The mechanism of plant tolerance to herbivore damage is often associated with compensatory regrowth, reallocation of resources and the utilization of storage reserves (Fornoni 2011; Li et al. 2012). It has been observed that many plant species have storage organs located in a safe place, such as below the ground, and can tolerate tissue loss to herbivory through compensatory regrowth using this stored material (McNaughton 1983; Simons and Johnston 1999; Utsumi and Ohgushi 2007). The root system is responsible for the supply of water and inorganic nutrients; it is free of above ground herbivory and has been found to be strongly associated with the plant regrowth ability (van der Meijden et al. 1988; Marschener 1998; Wise and Abrahamson 2005). Since the storage of resources for regrowth, growth and investment in defense all draw from the same pool, they actually compete with each other for these limited resources (de Jong and Van Der Meijden 2000). Regrowth ability is therefore expected to be a trade off with growth (van der Meijden et al. 1988).

To be able to regrow after foliar herbivory, plants need to have stored a certain amount of carbohydrate in their roots to be used to regenerate new leaf tissue after defoliation. Many species rely upon large root systems that are speculated to have high levels of storage of nutrients for regrowth (Donaghyē and Fulkersonǵ 1998; Sosnová and Klimešová 2009; Chen et al. 2013; McCormick et al. 2013; Aranjuelo et al. 2015). However, roots have multiple functions: structural maintenance, nutrient and water retrieval for growth and storage of resources. Hence, a large root to shoot ratio may result from high storage levels but can also be the consequence of low nutrient availability (Brouwer 1983; van der Meijden et al. 2000). Thus, to understand regrowth ability and the role of the size of the roots in regrowth it is essential to study both the root storage and root size.

In this study we hypothesized that invasive plants may tradeoff their regrowth ability with growth as a response to the change in herbivore guilds in the introduced range. To test this we chose *Jacobaea vulgaris* as a model species. We compared plant growth, regrowth, and the underlying regrowth traits (root carbohydrate storage) between native and invasive *J. vulgaris* before and after an artificial defoliation. Since the selection pressure of specialist herbivores was absent throughout the invasive range during the first 100 years of introduction, a parallel evolution in *J. vulgaris* plants could be expected among their geographically and climatologically distinct invasive ranges. To our knowledge, this study is

one of the first that focuses on the comparison of regrowth ability and its underlying traits between the same plant species from invasive and native areas. Hence the result would contribute to the critical evaluation of the role of regrowth involved in the evolutionary shift mechanism behind invasion success.

Material and methods

Study species

Common ragwort, *Jacobaea vulgaris*, formerly known as *Senecio jacobaea*, is a monocarpic perennial plant that belongs to the family of the *Asteraceae*. It is native to Eurasia and was introduced into parts of Australia (Harper and Wood 1957), New Zealand (Poole and Cairns 1940) and the Eastern North America in the 1850s and into the Western North America in the 1900s (Harris et al. 1971). The populations of *J. vulgaris* from West and Eastern North America are geographically isolated since they are close to the coasts. The level of genetic variation of native *J. vulgaris* populations is similar among the different invasive ranges, suggesting that introductions from multiple source populations have occurred (Doorduin et al. 2010). Using an assignment analysis, the same study has shown that populations from the western coast of Europe are most likely the source populations.

Due to its content in pyrrolizidine alkaloids, *J. vulgaris* has received a pest status in introduced areas since these alkaloids are toxic to horses and cattle and has caused significant livestock losses (Johnson et al. 1985; Coombs et al. 1996; Stegelmeier et al. 1999; Gardner et al. 2006). In the native range, *J. vulgaris* is attacked by more than 70 herbivores but most herbivory is due to two specialist herbivores: *Tyria jacobaeae* (Cinnabar moth) and *Longitarsus jacobaeae* (a flea beetle) (Harper and Wood 1957; Joshi and Vrieling 2005). In North Western Europe, especially dune populations of *J. vulgaris* suffer a complete defoliation once every 2 or 3 years in mid-June by *T. jacobaeae* larvae. This defoliation is followed by herbivory by *L. jacobaeae* during August that also causes a large leaf loss (Windig and Vrieling 1996; van der Meijden et al. 2000). This native *J. vulgaris* shows a strong regrowth after complete defoliation (Islam and Crawley 1983; van der Meijden et al. 1988). On the other hand, in the USA, an introduced range, *J. vulgaris* has been observed to be fed upon by more than 40 species of generalist arthropods but no specialist herbivores (Frick 1972). In a common garden experiment, Joshi and Vrieling (2005) found that invasive *J. vulgaris* had, on average, a 12% lower regrowth capacity than genotypes from the native areas. In the past few decades, *T. jacobaeae* and *L. jacobaeae* have been introduced as biological control agents into the invasive range (Syrett 1983; McEvoy et al. 1991; McEvoy and Coombs 1999; McLaren et al. 2000). However, so far no evolutionary adaptation of invasive *J. vulgaris* populations has been observed after their exposure to these biological control agents (Rapo et al. 2010).

Plant material and growth conditions

Seeds were collected from 18 native populations in Europe and 18 invasive populations in Australia, West and Eastern North America (see Supplementary Table S1 and Supplementary Fig.S1 for detailed population information). Native seeds from the potential source

populations along the western coast of Europe were intentionally selected to compare them with invasive populations (Doorduin et al. 2010). For each population, seeds from three different plants (hereafter referred to as "mother plants") were germinated in petri dishes with moistened filter paper. After 5 weeks, 4 well-grown seedlings from each mother plant were selected and randomly assigned to the control treatment (control T0: seedling harvested at T0 before potting; control T1: plants harvested at T1; control T2: plants harvested at T2) and the clipping treatment (clipping T2: Plants defoliated at T1 and harvested at T2) as shown in Fig. 1. Therefore there were 108 plants for each treatment (1 seedling * 3 mother plants * 36 populations). Seedlings from control T0 were harvested and oven-dried for 3 days at 50°C and then weighed. Plants from control T1, T2 and clipping T2 were transplanted into 1 L pots with 20% potting soil (Slingerland potground, Zoeterwoude, The Netherlands), 80% sandy soil (collected from Meijendel, The Netherlands, 52º13'N, 4º34'E) and 2.5g Osmocote slow-release fertilizer (Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA; N:P:K:MgO 15:9:11:2.5). Plants were grown in a climate room at 20°C, 70% humidity, 16 hours daylight with a light intensity of 113 umol PAR $m^{-2}s^{-1}$. After 8 weeks, plants from control T1 were harvested. Meanwhile, plants from the clipping treatment were defoliated by removing the shoot part (1cm above the root crown) with a pair of scissors. The shoots were dried in an oven at 50°C for three days and weighed to determine their dry mass. As expected, the shoot dry mass removed at clipping T1 did not differ from the shoot dry mass of the harvested plants at control T1 for both native and invasive populations (ANOVA, native: $F_{1,34} = 0.124$, $p>0.05$; invasive: $F_{1,34} = 0.146$, $p>0.05$). This indicates that plant growth rates from control T1 and clipping T1 were similar before the moment of clipping and therefore we assumed there was no difference in the root biomass.

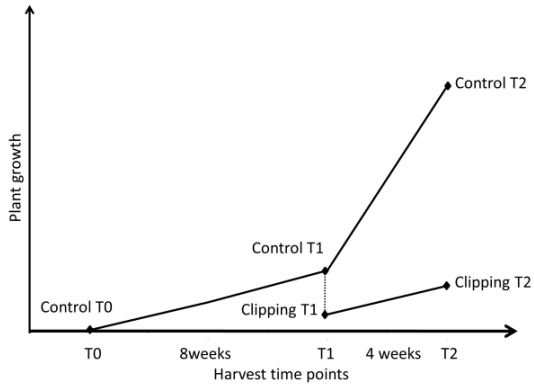

Fig. 1 Experimental design for the control treatment and clipping treatment and the harvest time points in this study. For the control treatment, plants were harvested at T0, T1 and T2. For the clipping treatment, plant shoots were clipped at T1 and plants were harvested at T2.

Subsequently, control T2 and clipping T2 were allowed to grow for a further 4 weeks till their final harvest after which their dry mass was determined. The samples were dried as described previously and the dry mass of shoots, roots, and total dry mass was determined. Except for control T0, the total leaf area of each plant from the other three treatments was measured at harvest using a portable leaf area meter (LI‑3100, LICOR, Inc., Lincoln, NE, USA). Four of the plants before clipping and six plants after clipping showed detrimental growth during this experiment and were not harvested.

Root inulin concentration (Carbohydrate storage)

Inulin is the natural carbohydrate storage source found in roots of the *Asteraceae* (Tertuliano and Figueiredo-Ribeiro 1993). The concentration of root inulin was measured as the difference between the free sugar content before hydrolysis and the total sugar content after hydrolysis. For each plant, 0.1 g of finely powdered root material was incubated with 4 mL distilled water at 80°C for 1 hour. After centrifugation, 1 ml of the supernatant was mixed with 2mL of 3,5-dinitrosalicylic acid (DNS, an aqueous solution of 0.03 mol•L^{-1}). The absorbance of this solution was recorded with a spectrophotometer at 540 nm and the concentration of free sugar was calculated using a calibration curve made with D(-) fructose according to Miller (1959). After this, another 1 mL of the same sample was hydrolysed with 200µl of inulinase (Novozym®960, Sigma-Aldrich) for 1 hour at 60 °C and the concentration of total sugar was measured by the same DNS method. The total inulin content (mg) in the root was calculated (=the root inulin concentration (mg•g^{-1}) ×total root dry mass (g)).

Since roots are used both for storage and to maintain the structure and take up nutrients and water, we divided the total root dry mass into the storage root dry mass (=root inulin content) and the structural root dry mass (=total root dry mass – root inulin content). Additionally, we calculated the root inulin-structural root ratio (=root inulin dry mass in the root/ structural root dry mass) and shoot to structural root ratio (=shoot dry mass/structural root dry mass) as indicators for regrowth ability and growth ability respectively. A separate analysis showed that inulin content in the shoots was below 10% of the root and did not differ between native and invasive genotypes (Data not shown).

Since there were two controls, the regrowth ability was calculated in two ways, based either on the control T1 or on the control T2. Regrowth ability T2/T1was calculated as the total dry mass from clipping T2 after regrowth/the total dry mass from the control T1. This calculation reflects the relative growth rate after clipping. Regrowth ability T2/T2 was calculated as the total dry mass from clipping T2 after regrowth/the total dry mass from the control T2 according to van der Meijden et al. (2000).

Climatic conditions

To examine the difference in the local climate among the four geographic regions (Europe, Australia, West and Eastern North America), 19 bioclimatic variables of the current conditions (ca 1950–2000) were downloaded from the WorldClim dataset (http://www.worldclim.org/current) in 5 arc-minutes resolution for each sampled population. A partial least square-discrimination analysis (PLS-DA) was performed with the SIMCA-P software (v.11.0, Umetrics, Umeå, Sweden) to classify all sampled populations based on the 19 bioclimatic variables. The scaling method for PLS-DA was unit-variance and the model was validated using the permutation method through 20 iterations.

Statistical analysis

As the main interest of this study was to find differences between invasive and native populations, statistical analyses were performed for all the measured traits in the three

treatments with nested ANOVAs, using origin (native-invasive) as the fixed factor, and region nested with origin and population nested within region as random factors. Normality of the residuals was corroborated with a Kolmogorov-Smirnov test. To obtain normality, shoot-structural root ratio and root storage-structural root ratio were log transformed. Additionally, in order to examine the differences among the native and three invasive regions (Australia, West and Eastern North America), a post hoc LSD test was used for any traits that significantly differed in any of the treatments between invasive and native populations.

Both regrowth ability estimates were tested with the same nested ANOVAs, with origin (native-invasive) as the fixed factor and region nested with origin and population nested within region as random factors. An additional post hoc LSD test was used to examine the differences between the invasive and native regions.

In order to test the effects of the initial root storage dry mass and the structural root dry mass at the moment of clipping on the net gain in dry mass 1 month after regrowth, a multiple regression analysis was conducted using the average net gain in dry mass per population (= total dry mass per population at clipping T2-root dry mass per population at clipping T1) as the dependent variable and the total inulin content and the structural root dry mass from each population at clipping T2 as independent variables. All analyses were carried out using SPSS 18.0 (SPSS: An IBM Company, Chicago, USA).

Results

Climatic conditions

The sampled invasive *J. vulgaris* populations of three geographically distinct regions were clearly separated from each other and the native region based on the 19 bioclimatic variables in a PLS-DA analysis (Fig. 2).

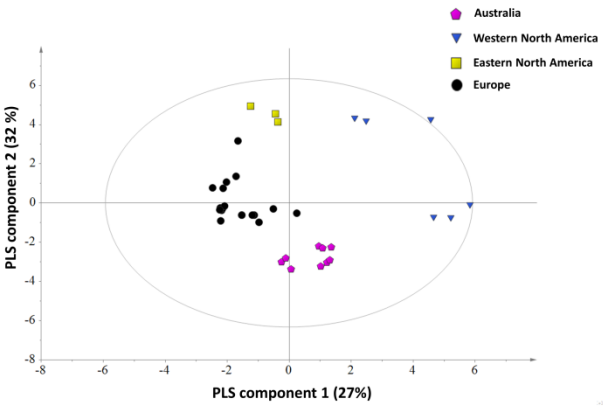

Fig. 2 Partial least square-discrimination analysis (PLS-DA) plots for the classification of the four geographic regions based on 19 bioclimatic variables from the collected site of each sampled population (N=18 for Europe, N= 9 for Australia, N=6 for Western North America and N=3 for Eastern North America).

Initial seedling mass

At the beginning of the experiment, there was no difference between the dry mass of seedlings of native and invasive *J. vulgaris* populations (Fig. 3, control T0, nested ANOVA: $F_{1,2}$ = 0.663, NS).

Growth in control T1

After 8 weeks of growth, invasive *J. vulgaris* populations on average had a 31% heavier shoot, a 20% smaller root, 50% higher total leaf area and 13% greater total dry mass than their native congeners in the control T1 (Table 1 and Fig. 3). When subtracting the mass of the storage component, i.e. inulin, from the total root dry mass, no difference was found between the structural root dry mass of invasive and native populations, but invasive populations had a 35% higher shoot-structural root ratio than native populations (Table 1). On average, the concentration and content of root inulin of invasive *J. vulgaris* genotypes was 25% and 38% lower respectively, resulting in a root inulin-structural root ratio that was 36% below that of native *J. vulgaris*.

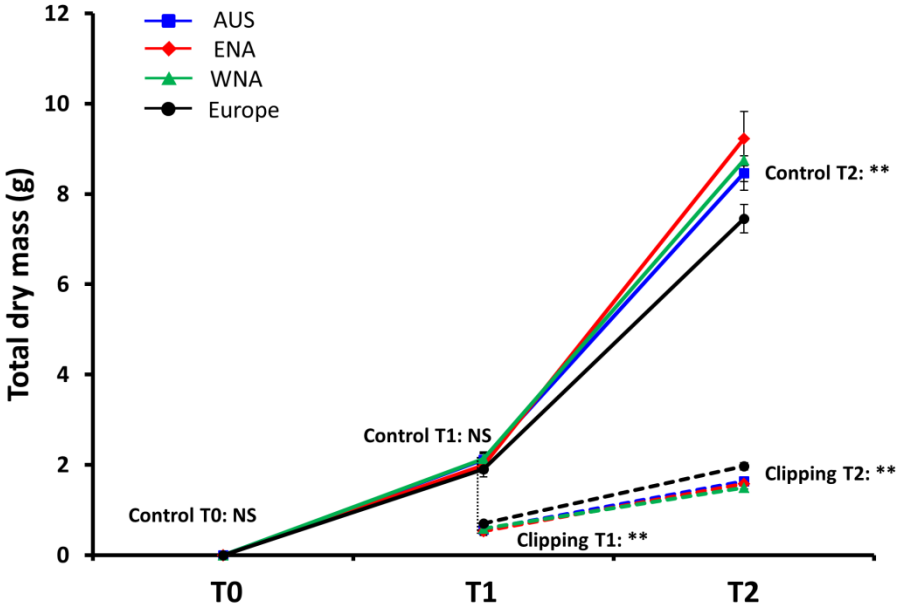

Fig. 3 The difference in total dry mass of *Jacobaea vulgaris* among invasive populations from three geographic regions (Australia, Eastern North America Western North America) and native populations from Europe at control T0, control T1, control T2, clipping T1 and clipping T2 respectively. Solid lines represent the control treatment and dashed lines represent the clipping treatment. Clipping T1 corresponds to the estimation of the root dry mass after clipping based on the root dry mass measured from control T1. The difference between origins and among regions at each treatment and at each time point was tested by a nested ANOVA, with origin (native versus invasive), region nested within origin and population nested within region as three fixed factors. The differences between origins are shown as ** (P≤0.01) and NS(not significant). There are no significant differences among regions.

Growth in control T2

After 12 weeks of growth without clipping, invasive *J. vulgaris* populations grew a 34% larger shoot, a 27% smaller root, had a 17% larger total dry mass and a 44% larger total leaf area than native populations in the control T2 (Table 1 and Fig. 3). Invasive populations had a 49% higher shoot-structural root ratio than native ones but did not differ in their structural root dry mass. In addition, invasive populations had, on average, 26% and 45% lower root inulin concentration and inulin content that resulted in 28% lower root inulin-structural root ratio than native populations.

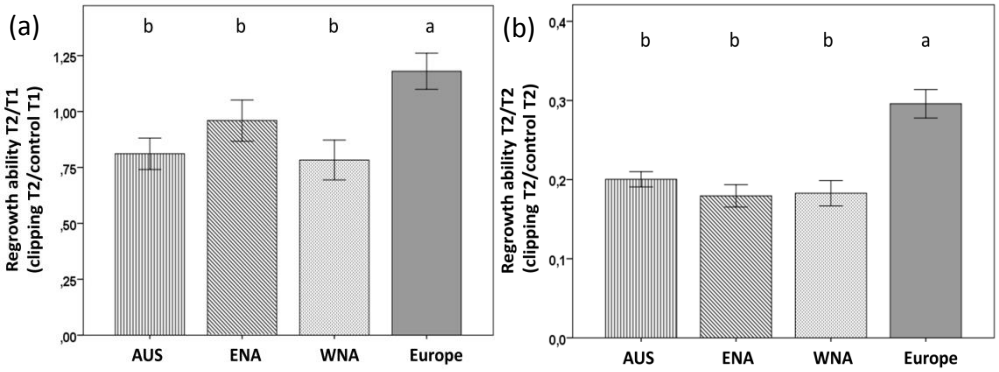

Fig. 4 The difference in regrowth ability T2/T1 (a) and regrowth ability T2/T2 (b) among invasive populations from three geographic regions (Australia, Western North America and Eastern North America) and native populations from Europe. Values are means ± SE. Different letters indicate significant differences among treatments at $p<0.05$ according to a post hoc LSD test (ANOVA).

Growth in clipping T2 after defoliation

While in the control treatment, plants from all three invasive regions had a higher dry mass than the plants from the native region, the reverse was true at 4 weeks after defoliation (Fig. 3). The invasive *J. vulgaris* populations had on average 20% and 18% smaller shoots and roots, respectively, as well as 20% less total leaf area, resulting in a 20% lower total dry mass than the native *J. vulgaris* submitted to the clipping treatment (Table 1 and Fig. 3, clipping T2). Invasive populations had a 12% lower shoot-structural root ratio than native populations although they did not differ in structural root dry mass. No differences in root inulin concentration and root storage-structural root ratio were found but invasive populations had 27% less root inulin content than native ones.

Regrowth ability

Since there were two control treatments, two corresponding calculations were used to compare the regrowth ability of invasive and native *J. vulgaris* populations. The regrowth ability T2/T1 was calculated as the ratio between the total dry mass of plants from clipping T2 and the total dry mass of plants from control T1. The regrowth ability T2/T2 was calculated as the ratio between the total dry mass of plants from clipping T2 and the total dry mass of plants from control T2. The results showed that the invasive populations had, on

average, a 29% and 34% lower regrowth ability T2/T1 (0.82 vs 1.15, $F_{1,2}$=8.861, p=0.005) and regrowth ability T2/T2 (0.19 vs 0.29, $F_{1,2}$=21.320, p<0.001) respectively, than native populations (Fig. 4).

Furthermore, a multiple regression analysis showed that the root inulin content at the control treatment T1 positively correlated with plant net gain dry mass after one month regrowth while the structural root was negatively correlated (Fig. 5).

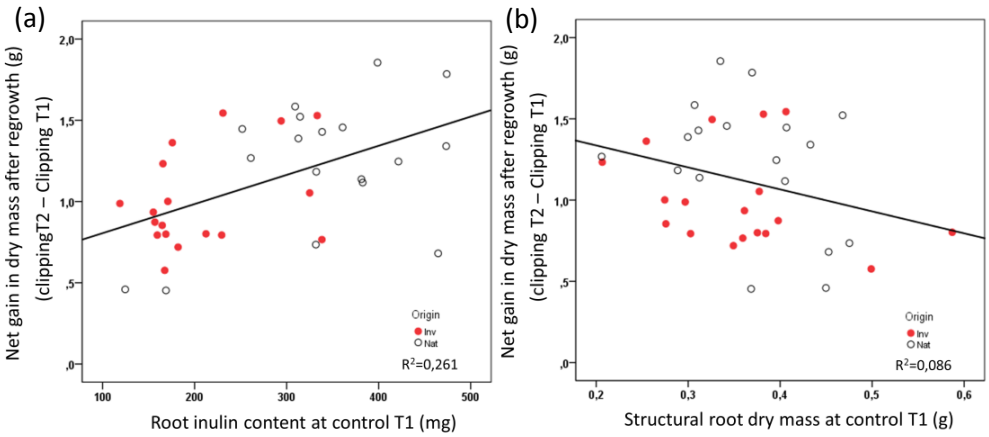

Fig. 5 Scatter-plots of the average net gain in dry mass per population from the clipping treatment after regrowth against the average root inulin content (a) and the structural root dry mass (b) of the same population at the moment of clipping (control T1). Multiple regression, n=36, total inulin: t=4.194, p<0.001; structural root dry mass: t= -2.810, p=0.008).

Parallel evolution

All studied traits that significantly differed between invasive and native populations, the invasive *J. vulgaris* populations from the three geographically distinct regions changed in the same magnitude and direction and all differed significantly from native populations (Table. 1, Fig. 3, 4 and 6).

Discussion

Invasive *J. vulgaris* populations showed a better growth performance, producing higher total mass, larger total leaf area and developing a higher shoot-structural root ratio than native populations in the control treatment at the final harvest (Table 1 and Fig. 3, T2). This trend was already evident at the moment of clipping (Table 1 and Fig. 3, T1). This is in line with a previous study by Joshi & Vrieling (2005) who, in a common garden experiment, found that after eight months of growth, invasive *J. vulgaris* plants had a higher vegetative growth and a 37% higher reproductive output than native plants. Several other invasive plant species have been found to exhibit superior growth ability than their native congeners as predicted by the EICA hypothesis (Daehler 2003; van Kleunen et al. 2010). Conversely, in our study, native populations performed better after 4 weeks of regrowth following simulated

herbivory by complete defoliation of the shoot. This also coincides with the results from the same study by Joshi and Vrieling (2005) who observed a 12% higher regrowth of native *J. vulgaris* genotypes after complete defoliation by clipping. Furthermore, Lin et al. (2015) found that native *J. vulgaris* populations regrew better than native populations after herbivory either by *Mamestra brassicae* or by *T. jacobaeae* under an intra-specific competition conditions. This indicates that native *J. vulgaris* are better adapted to defoliation than invasive ones. Apparently this comes at the cost of a reduced growth rate because in the invasive areas where *J. vulgaris* does not have to cope with severe defoliation by specialist herbivores they have lost part of their regrowth ability but attained higher dry masses.

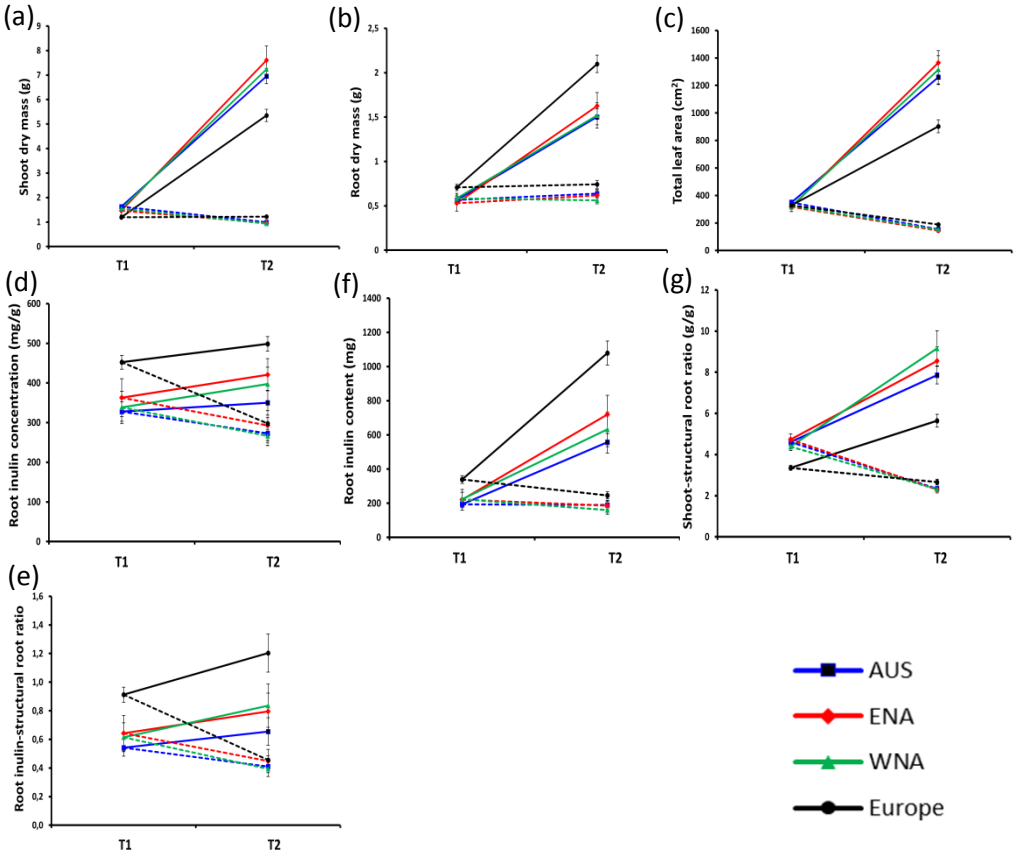

Fig. 6 The difference in shoot dry mass (a), root dry mass(b), total leaf area (c), root inulin concentration (d), root inulin content (e) shoot-structural root ratio (f) and root inulin-structural root ratio (g) among invasive populations from three geographic regions (Australia, Western North America and Eastern North America) and native populations from Europe at control T1, control T2 and clipping T2 respectively. Values are means ± SE. Solid lines represent the control treatment and dashed lines represent the clipping treatment.

Table 1 The differences of growth and regrowth traits between invasive and native *Jacobaea vulgaris* populations at control T1, control T2 and clipping T2. P values are from a nested ANOVA, with origin (native versus invasive) and population nested within origin as fixed factors, df=1, 72. P (origins): significance level of nested ANOVA between invasive and native origins. P (regions): significance level of nested ANOVA among regions. P (populations): significance level of nested ANOVA among populations. NS= not significant.

Variables	***Origin***	***Control T1***				***Control T2***				***Clipping T2***			
		Mean ± SE	***P (origin)***	***P (region)***	***P (population)***	***Mean ± SE***	***P (origin)***	***P (region)***	***P (population)***	***Mean ± SE***	***P (origin)***	***P (region)***	***P (population)***
Total leaf area (cm^2)	invasive	468,58±18,42	<0,001	NS	0,017	1295,60±45,60	<0.001	NS	0,858	152,03±6,57	0,014	NS	0,014
	native	312,52±11,78				902,73±47,77				189,21±9,57			
Shoot dry mass (g)	invasive	1,57±0,06	0,001	NS	NS	7,16±0,22	<0,001	NS	NS	0,974±0,032	0,001	NS	NS
	native	1,20±0,05				5,35±0,25				1,225±0,050			
Root dry mass (g)	invasive	0,57±0,03	0,007	NS	NS	1,53±0,07	0,001	NS	0,033	0,611±0,029	0,045	NS	NS
	native	0,71±0,04				2,10±0,10				0,743±0,045			
Total dry mass (g)	invasive	2,14±0,09	NS	NS	NS	8,69±0,27	0,01	NS	NS	1,58±0,06	0,003	NS	NS
	native	1,90±0,08	-0,061			7,45±0,32				1,97±0,09			
Structural root (g)	invasive	0,356±0,017	NS	NS	NS	0,919±0,043	NS	NS	NS	0,431±0,018	NS	NS	NS
	native	0,369±0,017				1,021±0,052				0,497±0,025	-0,072		
Shoot- structural root ratio (g/g)	invasive	4,55±0,13	<0,001	NS	0,039	8,41±0,38	<0,001	NS	NS	2,33±0,06	0,045	NS	NS
	native	3,36±0,12				5,64±0,32				2,66±0,14			
Root inulin concentration (mg/g)	invasive	337,55±20,07	<0,001	NS	NS	377,71±21,96	0,002	NS	NS	274,34±15,42	NS	NS	NS
	native	452,29±17,73				498,69±18,96				297,64±15,35			
Root inulin content (mg)	invasive	209,00±19,84	<0,001	NS	NS	609,32±49,34	<0,001	NS	NS	179,69±15,11	0,049	NS	NS
	native	339,00±22,64				1078,82±71,57			-0,053	246,11±22,40			
Root inulin-structural root ratio (g/g)	invasive	0,585±0,050	<0,001	NS	NS	0,739±0,073	0,016	NS	NS	0,412±0,031	NS	NS	NS
	native	0,912±0,05				1,204±0,136				0,456±0,031			

Our data further suggested that the increased regrowth ability is strongly associated with the amount of root carbohydrate storage. We found invasive *J. vulgaris* populations stored significantly less inulin in the root than native populations. Several studies have reported that the root non-structural carbohydrate storage plays an important role in regrowth after defoliation in several plant species (Ta et al. 1990; Corre et al. 1996; Avice et al. 1997; McCormick et al. 2013; Janeček and Klimešová 2014). Interestingly, we found that on average, native *J. vulgaris* plants reduced their amount of available inulin for their regrowth by 65 mg more than invasive *J. vulgaris*. The average relative growth rate of the control native plants between T1 and T2 is 0.049 $g \bullet g^{-1} day^{-1}$. The excess of 65 mg inulin used by the native *J. vulgaris* for growth should result in a net gain of 255 mg in plant dry mass after 4 weeks of growth equalling the difference in dry mass found between native and invasive *J. vulgaris* individuals at T2 of 250 mg (Table 1). This is in line with the multiple regression analysis that showed that the final dry mass of regrowth was positively influenced by the total root inulin content at the moment of clipping (Fig. 5). Therefore our results showed strong evidence that the regrowth performance of *J. vulgaris* depended on the root inulin storage and invasive *J. vulgaris* had been selected to store less inulin resulting in a poorer regrowth ability.

In general, previous studies have considered the ratio between total root and total shoot as an indicator of storage or regrowth (van der Meijden et al. 1988; Iwasa and Kubo 1997; Strauss and Agrawal 1999). Surprisingly, in contrast to the root inulin content, we found that the plant regrowth ability was negatively influenced by the structural root dry mass (Fig. 5). Maintaining a large amount of structural root might be costly, especially when plants suffer from shoot damage and require energy for later regrowth. Therefore a large root does not necessarily translate into better regrowth ability, and in turn the size of the structural root may even affect plant regrowth after damage negatively. We suggest that it is crucial to study the root storage and structure components separately rather than associate the entire root with plant regrowth ability.

A model was proposed by de Jong and Van Der Meijden (2000) which suggested that under repeated disturbance such as the outbreak of a specialist herbivore, plant genotypes that allocate more resources to storage should not suffer much and recover quickly through regrowth since a smaller fraction of its total biomass is removed by herbivory. This may explain why native *J. vulgaris* showed a better regrowth performance than their invasive congeners after clipping and we also did find that they developed larger roots and increased their inulin storage. We believe that most likely the selection pressure by the specialist herbivore *T. jacobaeae* in the native range plays an important role in the evolution of increased regrowth ability of native *J. vulgaris*. The foliar-feeding larvae of this specialist herbivore regularly defoliate all the aboveground parts of *J. vulgaris* in the native range during mid-June. Van der Meijden et al. (1988) showed that this univoltine specialist herbivore has broken through all plant defenses and that *J. vulgaris* uses regrowth as a strategy to overcome defoliation (Dempster 1971; van der Meijden et al. 1988). We therefore are of the opinion that the higher shoot-structural root ratio we found in the non-defoliated invasive *J. vulgaris* populations in our experiment represents a redistribution of resources from root storage to aboveground parts, thus contributing to better growth performance.

Our results strongly suggest that parallel evolution has occurred in the invasive *J. vulgaris* populations from the three geographically and climatically distinct invasive regions. It is noteworthy that there are alternative explanations e.g. bridgehead or anthropogenically induced adaptation to invasive regions (Lombaert *et al.* 2010; Hufbauer *et al.* 2012). However, genetic analyses showed that multiple introductions have most likely occurred in the invasive ranges (Doorduin *et al.* 2010), implying that the changes in the traits can be best explained by parallel evolution in the invasive *J. vulgaris* populations from the three geographically distinct regions. Such parallel evolution is more likely due to the disappearance of selection pressures from specialist herbivores than to the adaptation to local abiotic factors after invasion since the major abiotic factor, the local climatological conditions, are significantly different among the three invasive regions.

In conclusion, in this study we found invasive *J. vulgaris* populations have been selected to evolve increased growth ability and decreased regrowth ability compared to native populations, due to the lower investment in root carbohydrate storage. These results support the Evolution of Increased Competitive Ability hypothesis and the Shifting defense hypothesis that contends that due to the absence of adapted specialist herbivores, a net gain is obtained for increasing growth by the invasive plants as they invest less in anti-herbivore defense. Moreover, it shows that plant regrowth ability after herbivory is strongly dependent on carbohydrate storage in the root while its structural component affects regrowth negatively. Additionally, all the studied traits measured in the invasive *J. vulgaris* populations from the three geographically distinct regions changed consistently in the same predicted direction. It can be explained by a parallel evolution in response to the absence of specialist herbivores after invasion since the climatological conditions were significantly different among these invasive regions.

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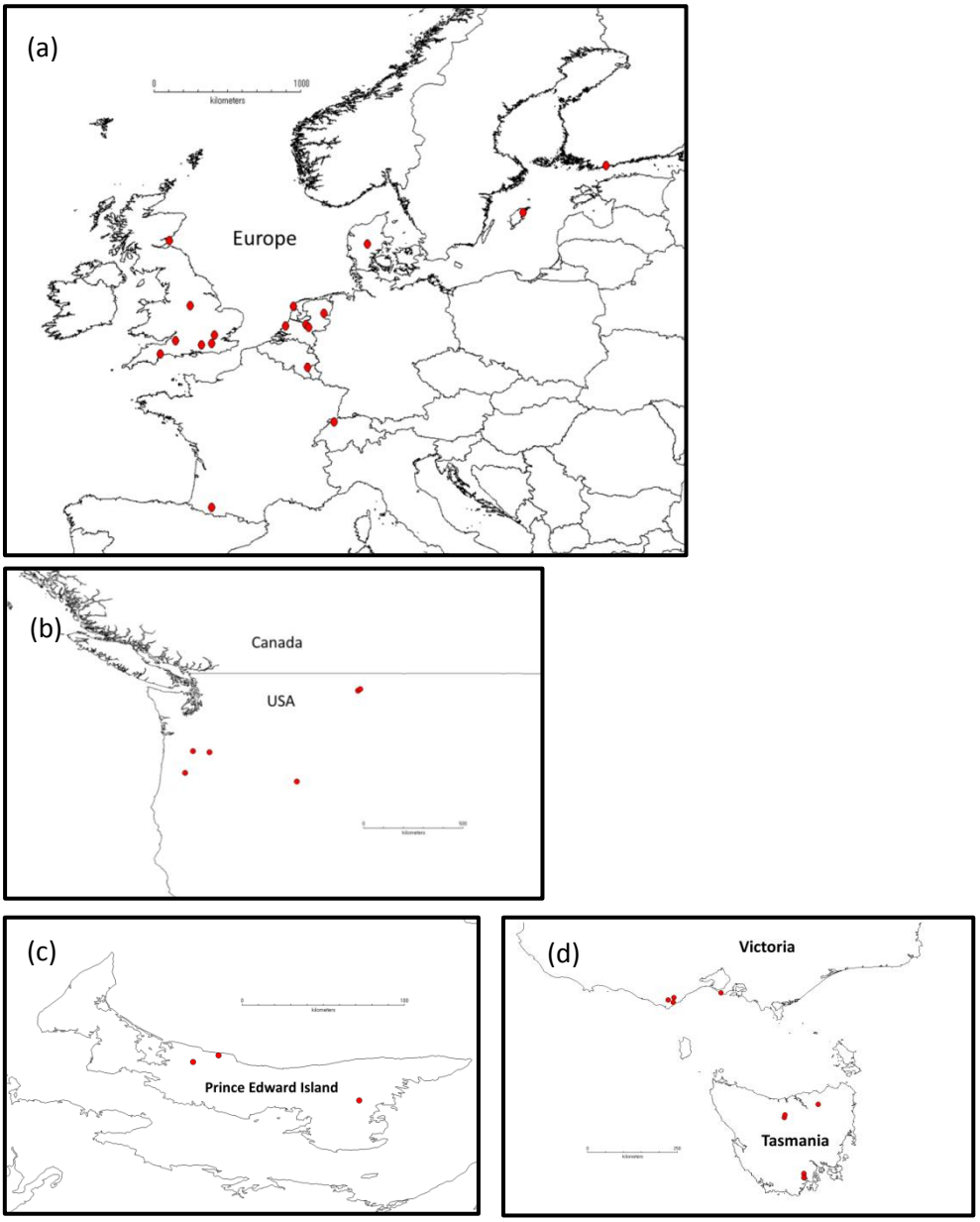
Supplementary Table S1 Origin of seeds of 18 native and 18 invasive populations of *Jacobaea vulgaris* used in this study

Origin	***country***	***Location***	***Longitude***	***Latitude***
Invasive	Australia	Franklin, Tasmania	146°19' E	41°33' S
		Dairy Plains, Tasmania	146°31' E	41°38' S
		Wild Dog Road, Victoria	143°40' E	38°41' S
		Beech Forest, Victoria	143°33' E	38°38' S
		Barramunga, Victoria	143°41' E	38°34' S
		Cape Schanck, Victoria	144°54' E	38°27' S
		Dairy Plains, Tasmania	146°32' E	41°34' S
		Targa, Tasmania	147°23' E	41°18' S
		Franklin, Tasmania	147°01' E	43°04' S
	Canada	Cardigan, Prince Edward Island	63°37' W	46°13' N
		Cavendish Beach, Prince Edward Island	63°24' W	46°29' N
		Clinton, Prince Edward Island	63°32' W	46°26' N
	USA	Indian Creek, Oregon	117°49' W	44°01' N
		West Crestmont, Oregon	121°51' W	45°22' N
		Island lake, Oregon	122°37' W	45°25' N
		Island Lake Road, Montana	114°59' W	48°20' N
		Kootenai National Forest, Montana	114°53' W	48°17' N
		Cochran Creek, oregon	122°58' W	44°25' N
Native	Belgium	Bertogne	05°40' E	50°05' N
	Denmark	Engesvang	09°21' E	56°10' N
	Finland	Inkoo, Kirkkonummi	24°00' E	60°02' N
	France	Lourdes	00°13' W	43°09' N
	Netherlands	Meijendel	04°20' E	52°07' N
		Wageningen	05°34' E	52°10' N
		Mossel	05°45' E	52°03' N
		Gees	06°41' E	52°44' N
		Texel	04°48' E	53°05' N
	Sweden	Sit Olofsholm, Gotland	18°54' E	57°43' N
	Switzerland	Mettembert	07°19' E	47°23' N
	UK	Alice holt forest	00°50' W	51°10' N
		Saint Andrews	02°47' W	56°20' N
		Derbyshire	01°30' W	53°07' N
		Sevenoaks weald	00°12' W	51°14' N
		Marshgreen	03°21' W	50°44' N
		Enfield	00°03' W	51°40' N
		Corston	02°26' W	51°23' N



Supplementary Figure S1 Distribution map of native and invasive *J. vulgaris* populations used in this study from Europe (a, n=18 populations), Western North America (b, n=6 populations), Eastern North America (c, n=3 populations) and Australia (b, n=9 populations)

Chapter 5

Parallel evolution in an invasive plant:

The effect of herbivores on competitive ability and regrowth of

Jacobaea vulgaris

Tiantian Lin, Peter G.L. Klinkhamer, Klaas Vrieling

Institute of Biology

Section Plant Ecology and Phytochemistry

Leiden University, PO Box 9505, 2300 RA Leiden

The Netherlands

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Abstract

A shift in the composition of the herbivore guild in the invasive range is expected to select for plants with a higher competitive ability, a lower regrowth capacity and a lower investment in defense. We show here that parallel evolution took place in three geographically distinct invasive regions that differed significantly in climatic conditions. This makes it most likely that indeed the shifts in herbivore guilds were causal to the evolutionary changes. We studied competitive ability and regrowth of invasive and native *Jacobaea vulgaris* using an intra-specific competition setup with and without herbivory. Without herbivores invasive genotypes have a higher competitive ability than native genotypes. The invasive genotypes were less preferred by the generalist *Mamestra brassicae* but more preferred by the specialist *Tyria jacobaeae*, consequently their competitive ability was significantly increased by the first and reduced by the latter. Invasive genotypes showed lower regrowth ability in both herbivore treatments.

Keywords

Biological invasions, evolution of increased competitive ability hypothesis, herbivore preference, herbivory, *Mamestra brassicae*, plant–insect interactions, ragwort, shift defense hypothesis, *Tyria jacobaeae*.

Introduction

Biological invasions have been reported as major threats to global biodiversity and can cause severe economic and ecological losses (Ehrenfeld 2010; Simberloff et al. 2013). In spite of their potential negative effects, invasive species provide an ideal opportunity for ecologists to study evolutionary changes by considering invasions as large scale experiments where major changes in selective forces have occurred. For invasive plants the most striking change is that of the herbivore guild, in particular the absence of specialist herbivores in the introduced ranges. After invasion, invasive species are freed from their native specialist herbivores though they are still under herbivore pressure by generalist herbivores and occasional specialist herbivores of congeneric plant species (Frick 1972; Castells et al. 2013). Such a shift in the herbivore composition towards a guild that is dominated by generalist herbivores is expected to exert altered selection on invasive plants that in turn leads to evolutionary changes in allocation patterns to defense and growth. Invasive species are therefore hypothesized to grow faster and show an increased competitive ability (Enemy Release Hypothesis (ERH), (Elton 1958; Keane and Crawley 2002), Evolution of Increased Competitive Ability hypothesis (EICA), (Blossey and Nötzold 1995) and the Shifting Defense Hypothesis (SDH) (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011).

So far evidence that changes in the herbivore guild are the selective force for changes in allocation patterns is largely circumstantial and other biotic or abiotic factors cannot be ruled out as being important. We therefore set out to study a system where multiple invasive regions are compared that differ in climatological conditions. If the change in the herbivore guild is the main selective force parallel evolutionary changes are expected in each of the geographically and climatologically differing invasive ranges.

Often a higher growth rate is used as a proxy for competitive ability (Grime 2006).However, since plant performance in the absence of competition might not necessarily translate into competitive ability, it is essential to examine plant competitive ability in a competitive environment (Bossdorf et al. 2004). To our knowledge, only a limited number of such studies have been conducted and the results are equivocal. Specifically, in five cases, invasive genotypes were the better competitors (Leger and Rice 2003; Ridenour et al. 2008; Zou et al. 2008; Beaton et al. 2011; Huang et al. 2012; Qin et al. 2012) while another three species showed opposite results (Vilà et al. 2003; Bossdorf et al. 2004; Franks et al. 2008) and two species showed no difference (McKenney et al. 2007; He et al. 2009). Partly these contradictory results may be caused by arbitrary choices of the interspecific competitors. To avoid associated problems with these arbitrary choices, Bossdorf et al. (2005) suggested to use an intraspecific competition setup in which plants from invasive and native populations compete against each other. This provides a direct method to test the competitive ability of native and invasive genotypes without the side effect of other factors such as local adaptation, species-specific interactions and possible co-evolution during plant invasion in nature (Bossdorf et al. 2004).

The outcome of competition experiments between native and invasive plants is also likely to depend strongly on whether or not herbivory is included and which herbivores are present

since the herbivore guild is changed in the introduced range. The Shifting Defense Hypothesis (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011) states that invasive plants are selected to be better defended against generalist herbivores and less well defended against specialist herbivores. As a result we predict that the competitive ability of invasive plants is higher than that of their native conspecifics without herbivores and that shifts in competitive ability depend on herbivore species.

Competitive ability is not only determined during the period of attack but also during the regrowth period thereafter. Since storing resources for regrowth is traded off with growth (van der Meijden et al. 1988), we expect that invasive plant genotypes are selected to have a reduced regrowth capacity because they suffer less from specialist herbivory. Specialist herbivores often defoliate plants completely while this is not often the case for generalist herbivores, therefore the competitive ability of native plants will increase under conditions that make it possible to regrow after herbivory. Allocation of resources to growth, storage for regrowth and defense are drawing from the same resource pool (de Jong and van der Meijden 2000) and all affect the competitive ability of plants, and therefore should be studied in combination when we examine the evolutionary changes in invasive plants. So far only one study has examined the competitive ability of invasive plants after regrowth following complete defoliation by different herbivores and found that competitive ability was not strongly affected by the type of herbivore (Huang et al. 2012). In their study differences between the effects of specialist and generalist herbivores on competition and regrowth ability may have gone unnoticed because in all treatments plants were completely defoliated and only the regrown biomass was studied. Studies on the effect of different herbivores and on competitive ability and regrowth of invasive plants are thus still lacking.

In this study we focus on the evolutionary change in competitive ability of an invasive plant and how herbivore species have shaped competitive ability using *Jacobaea vulgaris* (synonym *Senecio jacobaea*, Common ragwort). More specifically, we tested the effect of two herbivores that are likely to differ in the level of herbivory on the competitive ability of native and invasive *J. vulgaris* genotypes using a design in which the native and invasive genotypes competed with each other. We examined the competitive ability after attack and after allowing regrowth and ask (1) whether the invasive *J. vulgaris* genotypes have evolved better competitive ability than their native conspecifics; (2) whether the herbivore species affects the outcome of competition and (3) whether the invasive genotypes have evolved lower regrowth ability after herbivory. Moreover, since the selection pressure of specialist herbivores has been absent throughout the invasive range during the first 100 years of introduction, a parallel evolution is expected to occur in *J. vulgaris* plants among the three geographically and climatologically distinct invasive ranges. The present study uniquely combines the interactive effects of herbivory and regrowth using multiple invasive regions to provide a more comprehensive understanding behind the evolutionary changes of competitive ability in invasive plants under different types of herbivory.

Material and Methods

Study species

Jacobaea vulgaris is a monocarpic perennial species. It is native to Eurasia and was introduced into Australia (Harper and Wood 1957), New Zealand (Poole and Cairns 1940) and North America (Harris et al. 1971) in the 1850s. In the native range *J. vulgaris* is attacked by more than 70 herbivores and most damage is caused by two specialist herbivores: *Tyria jacobaeae* (Cinnabar moth) and *Longitarsus jacobaeae* (Tansy ragwort flea beetle) (Joshi and Vrieling 2005). In the USA, more than 40 species of generalist arthropods only were recorded to feed on it (Frick 1972). *J. vulgaris* contains pyrrolizidine alkaloids (PAs) as constitutive defense against generalist herbivores (Macel et al. 2004). In the native range, the specialist herbivores *T. jacobaeae* and *L. jacobaeae* sequester PAs for their own defense against predators and *T. jacobaeae* uses PAs as oviposition and feeding stimulants (Dobler et al. 2000; Macel and Vrieling 2003; Hartmann et al. 2004). In the last few decades, *T. jacobaeae* and *L. jacobaeae* have been introduced as biological control agents into to the invasive ranges (Syrett 1983; McEvoy et al. 1991; McLaren et al. 2000). So far no evolutionary adaptations of invasive J. vulgaris populations have been observed after the exposure to the biological control agents (Rapo et al. 2010). In a competitor-free, common garden experiment Joshi and Vrieling (2005) found that invasive *J. vulgaris* had higher vegetative and reproductive biomass than native genotypes and invasive genotypes were more susceptible to specialist herbivores but were better protected against generalist herbivores. All these findings indicated evolutionary shifts that may enable genotypes from the invasive range to have an increased competitive ability compared to native ones. Doorduin et al. (2010) found that the amount of genetic variation of native *J. vulgaris* populations does not differ from the different invasive ranges, suggesting that introductions from multiple source populations have occurred. Moreover an assignment analysis indicated that populations from the western coast of Europe are the most likely source populations.

Insect rearing

Specialist herbivore

In this study we used the univoltine *T. jacobaeae*, native to Europe and western central Asia, as a specialist herbivore. It has been introduced into New Zealand, Australia and North America (Dempster 1971).

Female moths of *T. jacobaeae* were collected in the dunes of Meijendel, The Netherlands, at the end of May, 2013. Moths were reared in a climate chamber (20°C, 50 to 70% RH, 16:8 h L/D). Eggs hatched within two weeks and larvae were reared on native *J. vulgaris* plants. For the experiment larvae originating from egg batches of 23 females were used.

Generalist herbivore

Mamestra brassicae (Cabbage moth) with a distribution ranges from Europe, Russia to Eastern Asia was used as a generalist herbivore. The larvae feed on a wide range of plants

and it is considered a pest. Adults can be found at any time from May to October due to the two or three overlapping generations (Chougule et al. 2008).

Eggs were collected from a lab culture of Wageningen University and hatched larvae were reared on *Brassica oleracea* in a climate chamber (20°C, 50 to 70% RH, 16:8 h L/D).

Experimental design

Plant growth conditions

Seeds of *J. vulgaris* were collected from 20 native populations from Europe and 20 invasive populations from Australia (9 populations), North America west coast (8 populations) and North America east coast (3 populations) (see Supplementary Table S1 and Supplementary Fig. S1). The populations on the west and east coast of North America are geographically isolated and molecular analyses suggest that their introduction is independent from the other invasive ranges (Doorduin 2012). We selected most native populations from the western coast of Europe which are potential source populations (Doorduin et al. 2010). In most comparable studies only a limited number of populations are included. This includes the risk that the results depend strongly on which particular populations are chosen. In this study we wanted to avoid such bias by including as many as possible populations from both ranges at the cost of an incomplete design. For each population seeds originating from three different (mother) plants were germinated in a petri dish. In total 60 mother plants were chosen for each origin (20 populations × 3 mother plants). For the competition experiment, we randomly paired 60 invasive mother plants with 60 native mother plants resulting in 60 replicates. Each replicate consisted of five well-grown individual seedlings (sibs) from both the invasive and native mother plant and these were divided over five 0.5 L pots consisting of 5 treatments: 2 monocultures treatments and 3 competition treatments with and without herbivores as shown in Fig. 1. In total there were 600 plants (2 origins × 5 treatments × 60 pairs). As *J. vulgaris* is self-incompatible and flowering takes place gradually over the composite flower head and hence over time it is anticipated that the seeds will be a mixture of full and half sibs.

Before planting fresh mass of the seedlings was measured. Seedling fresh mass did not differ between seedlings from the native and invasive range (ANOVA, $F_{1, 599}$ =0.830, p=0.368). A pilot study under the same conditions, using a range of densities showed that if two *J. vulgaris* individuals were planted in a 0.5 L pot, they showed a reduced yield compared to single plants (Supplementary Fig. S2). Each pot contained 10% potting soil (Slingerland, Zoeterwoude) and 90% sandy soil collected from Meijendel (52º13'N, 4º34'E), a site where ragwort occurs naturally. Plants were grown in a climate chamber (20°C, 70% RH, 16:8 h L/D) for 8 weeks at the Institute of Biology, Leiden, The Netherlands (52º09'N, 4º29'E), before the start of the experiment.

Climatic conditions

To exam the difference in local climate among the four geographic regions (Europe, Australia, Eastern North America, Western North America), 19 bioclimatic variables of the

current conditions (ca 1950–2000) were downloaded from the WorldClim dataset (http://www.worldclim.org/current) in 5 arc-minutes resolution for each sampled population.

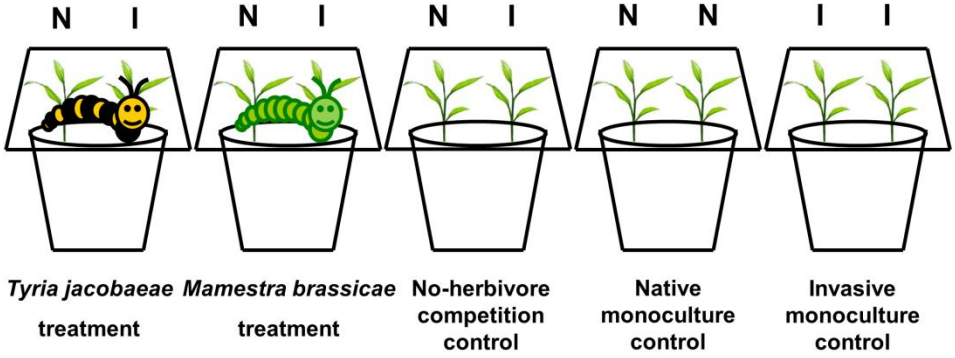

Fig. 1. Experimental design for the competition and monocultures for 1 replicate. Sibs were derived from 1 native and 1 invasive of *Jacobaea vulgaris* mother plant. N denotes sibs originating from one native mother plant, I denotes sibs originating from one invasive mother plant. All pairs were replicated 60 times with different sib families from different invasive and native populations.

A partial least square-discrimination analysis (PLS-DA) was performed with the SIMCA-P software (v.11.0, Umetrics) for classifying all sampled populations based on the 19 bioclimatic variables. The scaling method for PLS-DA was unit-variance and the model was validated by using the permutation method through 20 applications.

Herbivore bioassay

After 8 weeks, all pots in all treatments were fit through a hole into a 16 cm *12 cm *7cm cage with a transparent lid with gauze. To the specialist treatment, eight second-instar larvae of *T. jacobaeae* were released. To the generalist treatment, eight second-instar larvae of *M. brassicae* were released. In order to evaluate the potential impacts of caterpillar fresh mass on their feeding performance, a representative sample of 6 batches of 10 larvae of each species were weighed and no difference was found between the fresh mass of *T. jacobaeae* and *M. brassicae* (ANOVA, $F_{1,5}$ =1.228, p=0.284) at the start of the experiment. The no-herbivore competition control, the native and invasive mono-culture treatments without herbivores were all placed in the same type of cages as the herbivory treatments (Fig. 1). After 12 days of herbivory, larvae were removed and the total fresh mass of larvae in each pot was measured. The percentage biomass reduction of each leaf area per plant was estimated and recorded and half of the replicates in all treatments were selected and harvested taking care that from each of the three mother plants from a population one or two were left for the second harvest. Plant material was dried in an oven at 50 °C for 3 days and the shoot mass from each genotype was measured separately. Since the roots of both genotypes from the same pot could not be separated, the whole root mass was measured (1st harvest). From the remaining 30 replicates the cages were removed. Plants were grown for another 4 weeks and then harvested (2nd harvest).

Statistical analysis

The differences in final caterpillar fresh mass per pot between the two herbivores were tested using a Kruskal-Wallis test. Shoot biomass reduction by caterpillars on each genotype was calculated as the percentage biomass reduction of leaf area per genotype × the shoot biomass of the same genotype from the no-herbivory competition control. We compared the shoot biomass reduction of the herbivore treatments of invasive individuals from the three invasive ranges with ANOVA and then performed a separate ANOVA for the neighboring native individuals from the same pot. These two analyses are not entirely independent but we want to test whether the genotypes from the invasive ranges differ from each other and whether the neighboring native plants paired to the invasive ranges differ among the invasive ranges. Next a paired t-test on shoot biomass reduction was carried out to compare within a range invasive genotypes and their native neighbors.

In order to evaluate the competitive ability of native and invasive *J. vulgaris* genotypes, we represented the data as a replacement series combining the shoot dry mass of the native and invasive genotypes from the no-herbivore competition control and both native and invasive monoculture controls (De Wit 1960). A relative replacement rate >1 indicates that the invasive genotypes outcompete native genotypes while a relative replacement rate <1 indicated that native genotypes are more competitive. To test if curves in the replacement series were significantly convex or concave, the shoot yield of each invasive or native genotype in the mixture was tested against the average shoot yield of the same invasive or native monoculture by a paired t-test.

To compare the competitive ability of invasive and native genotypes between regions and populations, we used the difference in shoot dry mass of the invasive genotypes minus that of the competing native neighbors in the same pot. If native and invasive genotypes are equal competitors the difference on average would be zero. These differences of each competition treatment in each harvest were tested with a nested ANOVA, with invasive region and invasive population nested within in invasive region as random factors. Specifically we tested the intercept as this indicates if the difference in shoot dry mass is significantly deviating from zero and hence would indicate which genotype is the better competitor. A significantly positive intercept shows that the competitive ability of invasive genotypes is higher than that of the their native neighbors while a significantly negative intercept shows the reverse. The analysis above assumes that native populations do not play a role. Therefore shoot dry mass of invasive genotypes minus the shoot dry mass of native genotypes was tested with a separate ANOVA using native population as a fixed factor.

In order to further compare the competitive ability among three invasive regions (Australia, Eastern North America and Western North America), the relative replacement rate of invasive genotypes was calculated as shoot dry mass of invasive genotype / shoot dry mass of native genotype per pot, according to Williams & McCarthy (2001). The relative replacement rates among the three invasive regions from each treatment were tested with an ANOVA followed by a post hoc LSD test. To compare the relative replacement rate among different treatments, the average value of three invasive regions from each treatment was calculated and tested with a post hoc LSD test.

Since invasive motherplants from the same population were randomly paired with different native motherplants and each population in each harvest was represented by one or two motherplants, the regrowth ability of each population was calculated as the average shoot dry mass from the 2nd harvest / the average shoot dry mass from the 1st harvest of the mother plants from the same population. The differences in regrowth ability among four regions in each treatment were log transferred and tested with a post hoc LSD test.

The data that are not normally distributed were log transformed first and all analyses were carried out using SPSS 18.0 (SPSS: An IBM Company).

Results

Climatic conditions

The sampled populations of the three invasive regions (Australia, Eastern North America and Western North America) were clearly separated in a PLS-DA plot based on the 19 bioclimatic variables (Fig. 2).

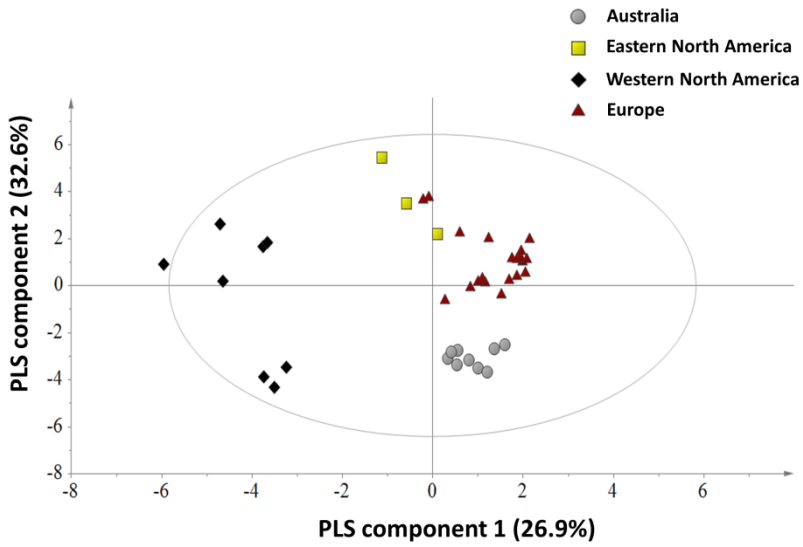

Fig. 2 Partial least square-discrimination analysis (PLS-DA) plot classifying the four geographic regions of native and invasive *Jacobaea vulgaris* populations based on 19 bioclimatic variables collected for each sampled population.

Herbivore performance

Average fresh mass of the specialist *T. jacobaeae* larvae per pot were doubled compared to the generalist *M. brassicae* larvae after 12 days feeding on leaves of *J. vulgaris* (1.25g vs. 0.59g, Kruskal-wallis test: n=120, χ^2=61.905, p<0.001) although larval mass at the start of the experiment was not significantly different between the species.

Total reduction of leaf biomass caused by the herbivores

Tyria jacobaeae reduced the shoot biomass of invasive genotypes twice as much as the native genotypes for all ranges (Fig. 3a). In contrast, *M. brassicae* treatment significantly reduced the shoot biomass of the native genotypes compared to all invasive ranges (Fig. 3b). There were no significant differences in shoot biomass reduction among the invasive genotypes from the three invasive regions (Australia, Eastern North America and Western North America) (Fig. 3).

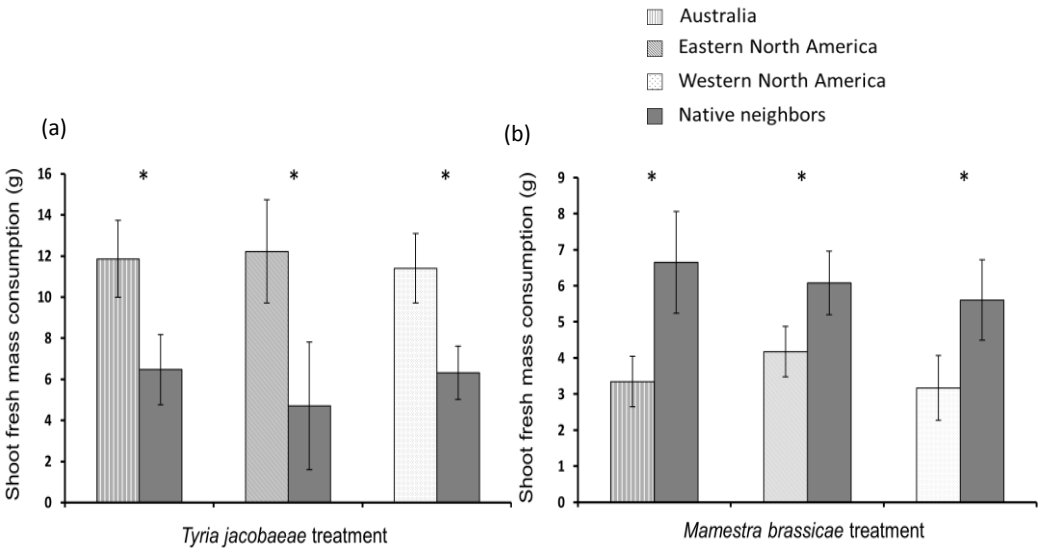

Fig. 3. Average shoot biomass reduction of the specialist herbivore *Tyria jacobaeae* treatment (a) and the generalist herbivore *Mamestra brassicae* treatment (b) on *Jacobaea vulgaris* genotypes from each invasive region (Australia, Eastern North America, Western North America) compared to their competing native genotypes after 12 days of herbivory. Values are means ± SE. The difference between invasive genotypes and their competing native genotypes per invasive range were tested with a paired *t*-test (*P*-value: *≤0.05). Difference among the three invasive regions for invasive and their competing native genotypes were tested by separate ANOVAs (a: invasive genotypes, $F_{2,59}$=0.843, p=0.436; native neighboring genotypes: $F_{2,59}$=0.717, p=0.492; b: invasive genotypes, $F_{2,59}$=0.078, p=0.952; native neighboring genotypes: $F_{2,59}$=1.300, p=0.343).

Competition

In the analyses of the difference in shoot dry mass between invasive and native genotypes in the same pot, invasive genotypes showed a higher competitive ability than native genotypes in both the no herbivory competition control and the *M. brassicae* treatment at both harvests indicated by significant positive intercepts (Table 1). Both invasive region as well as invasive population nested in region were non-significant and a separate ANOVA showed that native population did not affect the results (Supplementary Table S2).

Table 1 Summary of analyses of variance of the difference in shoot dry mass between invasive and native genotypes from the same pot in all the competition treatments and in both harvests. The differences in shoot dry mass were tested with a nested ANOVA, with invasive region and invasive population nested within invasive region as random factors which assuming that native population are randomly paired to invasive plants and its possible effects are cancelled out. The intercept tests if the difference between shoot dry mass of invasive minus that of the competing native genotypes deviates from zero and the average absolute difference with standard error is indicated in the first row of the table. **P<0.01, ***P<0.001.

		***Tyria jacobaeae* treatment**				***Mamestra brassicae* treatment**				***No-herbivory competition* control**			
		1st harvest		***2nd harvest***		***1st harvest***		***2nd harvest***		***1st harvest***		***2nd harvest***	
Source	***df***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***
Average difference (Invasive-Native) in shoot dry mass per pot		0.104±0.084		-0.589±0.301		0.948±0.118		0.950±0.274		0.707±0.120		1.852±0.559	
Intercept	1	0.387	1.998	6.766	1.982	18.766	57.766 ***	17.817	7.126 **	9.597	20.865 ***	76.175	8.053 **
Invasive region	2	0.077	0.401	0.697	0.202	0.61	1.875	1.31	0.522	0.082	0.178	2.908	0.308
Invasive population nested in Invasive region	17	0.189	0.696	3.701	2.438	0.315	0.649	2.579	1.303	0.462	1.083	9.362	0.927

Competition without herbivory

Depicted as a classical de Wit's replacement series, the invasive genotypes had a 43% and 56% higher shoot dry mass than the native genotypes in the absence of herbivores at the 1st and 2nd harvest respectively (Fig. 4, Table 2). Shoot yield of the native genotypes was significantly concave while that of the invasive genotypes was not convex at both harvests. As a result the yield total of shoot dry mass under competition was significantly lower than that of the two mono-culture controls (Fig. 4). The relative replacement rate of the invasive genotypes from the 1st harvest was on average 85% higher than the 2nd harvest while no significant difference was found among the three invasive regions at both harvests (Table 1 and Fig. 4, no-herbivore competition control).

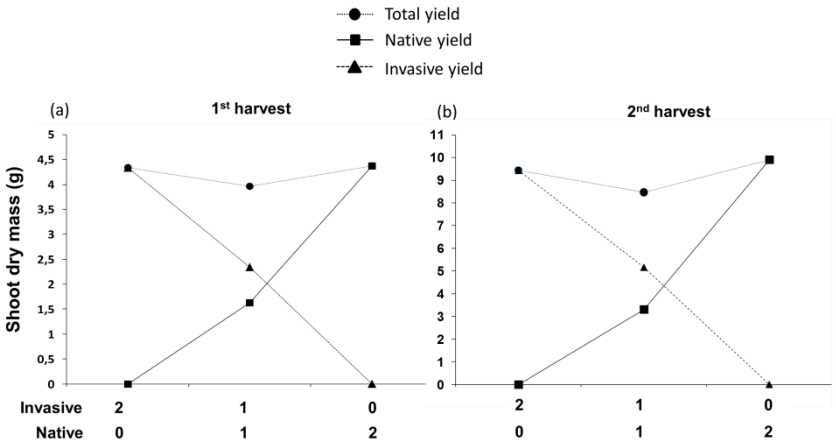

Fig. 4. Replacement series of the shoot dry mass from invasive and native *Jacobaea vulgaris* genotypes in the absence of herbivores (no-herbivore competition control, native and invasive monoculture controls) at the 1st harvest (a) and the 2nd harvest (b).The significance of the convex or concave trend of each invasive or native yield line was tested from the deviation of the average shoot yield of the same invasive or native monoculture by a paired t-test. For the 1st harvest, the native yield line is significantly concave (paired t-test, df=29, t=6.639,p<0.001) while the invasive yield line is not significantly convex or concave (paired t-test, df=29, t=1.329, p>0.05). For the 2nd harvest, the native yield line is significantly concave (paired t-test, df=29, t=5.170, p<0.001) while the invasive yield line is not significantly convex or concave (paired t-test, df=29, t=1.101, p> 0.05).

Competition with herbivory

We examined the effect of the specialist *T. jacobaeae* and the generalist *M. brassicae* on plant competition by comparing the shoot dry mass of invasive genotypes with native genotypes in each pot. Compared to the no-herbivore competition control, the average value of the relative replacement rate of the invasive genotypes was significantly reduced (50%) after consumption by the specialist *T. jacobaeae* while it increased by 34% after the consumption by the generalist *M. brassicae* at the 1st harvest (Fig. 5a, Table 2). After one month of regrowth, the average value of the relative replacement rate of the invasive genotypes over native ones became lower in both of the herbivore treatments compared to no herbivore control at the 2nd harvest (Fig. 5b, Table 2). This can be explained by the better

regrowth ability of the native genotypes compared to invasive genotypes after herbivore attack. In addition, the relative replacement rates of the invasive genotypes among the three geographic regions in both herbivore treatments consistently changed with the same magnitude and direction (Table 1 and Fig. 5).

Table 2 Results of a paired *t*-test on shoot dry mass between the invasive and native *jacobaea vulgaris* genotypes in treatment A, B and C to test for differences in performance between origins under different herbivore treatments at both harvests. Treatment A: herbivory by *Tyria jacobaeae*; Treatment B: herbivory by *Mamestra brassicae*; Treatment C: no-herbivory competition control.

Harvest time	***Treatment***	***Shoot dry mass (g)***		***Paired t-test***		
		Native	***Invasive***	***df***	***t-value***	***p-value***
1st Harvest	A	1.41±0.09	1.31±0.07	29	0.377	0.709
	B	1.23±0.07	2.18±0.10	29	7.429	<0.001
	C	1.63±0.08	2.34±0.09	29	5.915	<0.001
2nd harvest	A	2.49±0.15	1.90±0.20	29	2.188	0.037
	B	2.27±0.18	3.22±0.26	29	2.728	0.010
	C	3.31±0.28	5.16±0.42	29	3.311	0.002

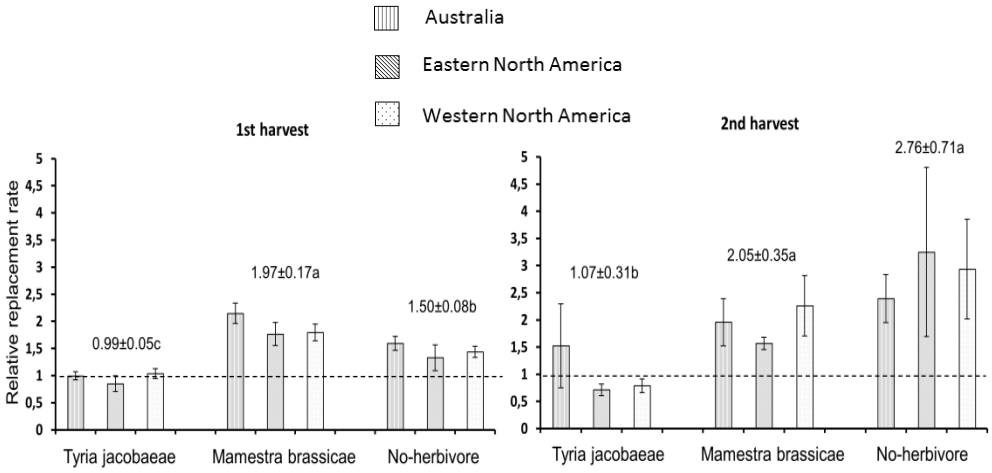

Fig. 5. Relative replacement rate of the invasive genotypes (= shoot dry mass of invasive genotype/shoot dry mass of native genotype per pot) from the three invasive regions (Australia, Western North America, Eastern North America) under three treatments at the 1st harvest (a) and at the 2nd harvest (b). Dashed line indicates a relative replacement rate =1. When the relative replacement rate >1 indicates that the invasive genotypes outcompete native genotypes. Relative replacement rate <1 indicated that native genotypes are more competitive. No significant differences were found among the three invasive regions within each treatment according to the post hoc LSD test. The average values of the relative replacement rate of each treatment are listed above the bars. Values are means ± SE. Different letters in the average value indicate significant differences among treatments at p<0.05 according to post hoc LSD test.

Regrowth

Native genotypes benefitted from their regrowth capacity after herbivory by showing significantly higher regrowth in dry mass than their invasive competitors for all invasive ranges after herbivory by the specialist *T. jacobaeae* and the generalist *M. brassicae* respectively (Fig. 6). No significant differences in regrowth ability among the three invasive regions (Australia, Eastern North America and Western North America) were detected.

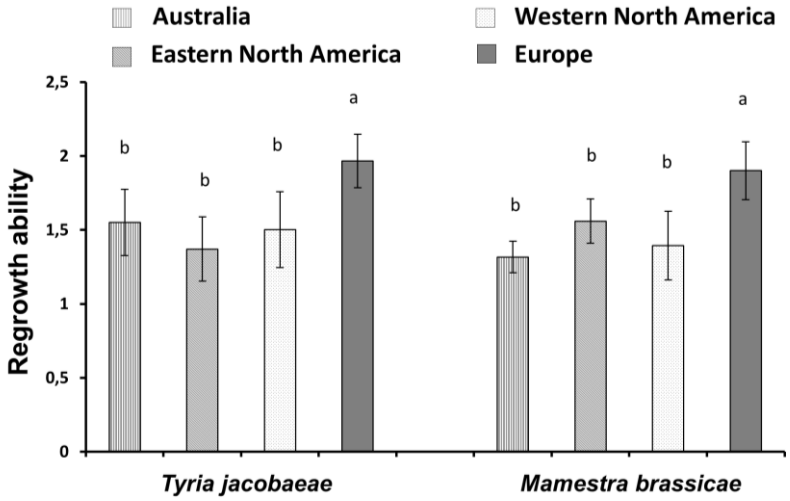

Fig. 6. Regrowth ability (= average shoot dry mass from the 2nd harvest / average shoot dry mass from the 1st harvest) of *Jacobaea vulgaris* populations from invasive regions (Australia, Western North America, Eastern North America) and native region (Europe) four weeks after specialist and generalist herbivores have been removed. Values are means ± SE. Difference among the four regions were tested by a post hoc LSD test. Different letters within each treatment indicate significant differences among regions at $p<0.05$.

Discussion

Parallel evolution among invasive regions

In this study we found that all traits measured (herbivore preference, competitive ability and regrowth ability) among invasive *J. vulgaris* genotypes from three geographically distinct regions consistently changed in the same magnitude and direction in each competition treatment and are all significantly different from their native neighbors (Fig. 3, 5 and 6) despite the fact that the local climate conditions differed among the three invasive regions (Fig. 2). This strongly suggests parallel evolution but there are alternative explanations e.g. bridgehead or anthropogenically induced adaptation to invasive regions (Lombaert et al. 2010; Hufbauer et al. 2012). However, genetic analyses showed that multiple introductions have occurred in the invasive ranges (Doorduin et al. 2010), suggesting that the changes in the traits can be best explained by parallel evolution in the invasive *J. vulgaris* genotypes in the three introduced regions. Such parallel evolution is most likely due to the disappearance

of selection pressures from specialist herbivores rather than the adaptation to local abiotic factors after invasion.

Competition without herbivory

The major prediction of the Evolution of Increased Competitive Ability hypothesis is that the escape from specialist herbivores leads to an evolutionary shift in energy allocation from defense to growth and consequently leads to a higher competitive ability in invasive plants. Here, we found, as predicted by the Evolution of Increased Competitive Ability hypothesis, that the invasive genotypes outcompeted their native conspecifics under herbivore-free conditions. The increased competitive ability of invasive *J. vulgaris* compared to native genotypes is in line with some studies (Leger and Rice 2003; Zou et al. 2008; Beaton et al. 2011; Huang et al. 2012; Qin et al. 2012) but contradictory to others (Vilà et al. 2003; Bossdorf et al. 2004; McKenney et al. 2007; Franks et al. 2008; He et al. 2009; Felker-Quinn et al. 2013). Bossdorf et al. (2004) suggested that selection for competitive ability is not only influenced by the potential trade-off with defense but also depends on the level of competition. If the level of competition is less in the invasive ranges, it may counteract or even reverse the effects of the trade-off with defense on selection for competitive ability. It is clear that is not the case for *J. vulgaris*.

Competition under specialist and generalist herbivory

In the native range, defenses of *J. vulgaris* plants are maintained at intermediate levels by the opposing selective pressures of specialist and generalist herbivores (van der Meijden 1996). But in the invasive range, the strong shift in herbivore complex towards generalist herbivores is expected to select for plants with defenses that act against generalist herbivores. As an extension of the Evolution of Increased Competitive Ability hypothesis, the Shifting Defense Hypothesis takes also the selection pressure imposed by generalist herbivore into account (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011). It predicts plants that in the invasive ranges plants defend themselves against generalist herbivores while they economize on defenses against adapted specialist herbivores. In this study, combining the results from the shoot biomass reduction with herbivore performance, it clearly shows that the invasive *J. vulgaris* genotypes were less preferred by the generalist herbivore *M. brassicae* but were more preferred by the specialist herbivore *T. jacobaeae* compared to their native conspecifics. The findings are consistent with a previous study on the same plant species (Joshi and Vrieling 2005). In their experiments, *M. brassicae* had higher mortality while *T. jacobaeae* showed a higher survival on invasive *J. vulgaris* genotypes. Their and our findings are in line with the Shifting Defense Hypothesis but our results cannot be seen as a full proof for the Shifting Defense Hypothesis since only one specialist and one generalist herbivore were used.

The Shifting Defense Hypothesis predicts that depending on the type of herbivore the outcome of competition between native and invasive plants are reversed. Here we show

that the effects of specialist and generalist herbivores are of key importance for the outcome of the competitive ability of *J. vulgaris* genotypes. The results showed that the treatment with the generalist *M. brassicae* significantly enhanced the competitive ability of the invasive *J. vulgaris* genotypes compared to the treatment without herbivory while the specialist *T. jacobaeae* decreased it. These findings are in line with the Evolution of Increased Competitive Ability hypothesis and Shifting Defense Hypothesis and show that herbivore species are playing a decisive role in shaping the competitive ability of invasive plants. As a consequence, the shift in the herbivore guild in the introduced range selected for invasive plants to have better competitive ability in the presence of generalist herbivores and hence contribute to their invasion success. More importantly, our results indicate that a competition experiment without generalist herbivores underestimates the competitive ability of plants from the invasive range.

Regrowth ability

Native genotypes had a significant higher regrowth ability than invasive genotypes after either the generalist *M. brassicae* or the specialist *T. jacobaeae* herbivory (Fig. 6). This is consistent with the finding of Joshi and Vrieling (2005) who found that native *J. vulgaris* genotypes had better regrowth after complete defoliation by clipping in a competition-free condition in a common garden experiment. The higher regrowth ability of native genotypes may be explained by a 51% higher polysaccharide storage in their roots compared to the invasive genotypes (Chapter 4) that can be used for regrowth (Saengkanuk et al. 2011). Most likely selection pressure by the specialist *T. jacobaeae* in the native range plays an important role. The foliar-feeding larvae of this specialist herbivore regularly defoliate all the aboveground parts of *J. vulgaris* in the native range while this is not the case for the herbivore guild that is dominated by generalist herbivores in the invasive range. Van der Meijden et al. (1988b) showed that the univoltine cinnabar moth has broken through all plant defenses and that ragwort uses regrowth as a strategy to overcome defoliation by the cinnabar moth (Dempster 1971; van der Meijden et al. 1988).

In conclusion, our results showed all the studied traits (herbivore preference, competitive ability and regrowth ability) measured in the invasive *J. vulgaris* genotypes from three geographically distinct regions changed consistently with the same magnitude and direction for all the treatments. It can be explained by a parallel evolution in response to the absence of specialist herbivores after invasion since the climatological conditions were significantly different among these invasive regions. Furthermore, we found that the invasive *J. vulgaris* genotypes had a higher competitive ability than native genotypes without herbivores but that the outcome of the competition is strongly depend on the type or level of herbivory. We clearly show that the invasive genotypes were less preferred by the generalist *M. brassicae* but more preferred by the specialist *T. jacobaeae* and as a consequence, the competitive ability of invasive genotypes was significantly increased by *M. brassicae* while it was reduced by *T. jacobaeae*. The data further suggested that the increased competitive ability of the invasive genotypes is accompanied by a decrease in regrowth ability. Our results support the Evolution of Increased Competitive Ability hypothesis and Shifting Defense Hypothesis.

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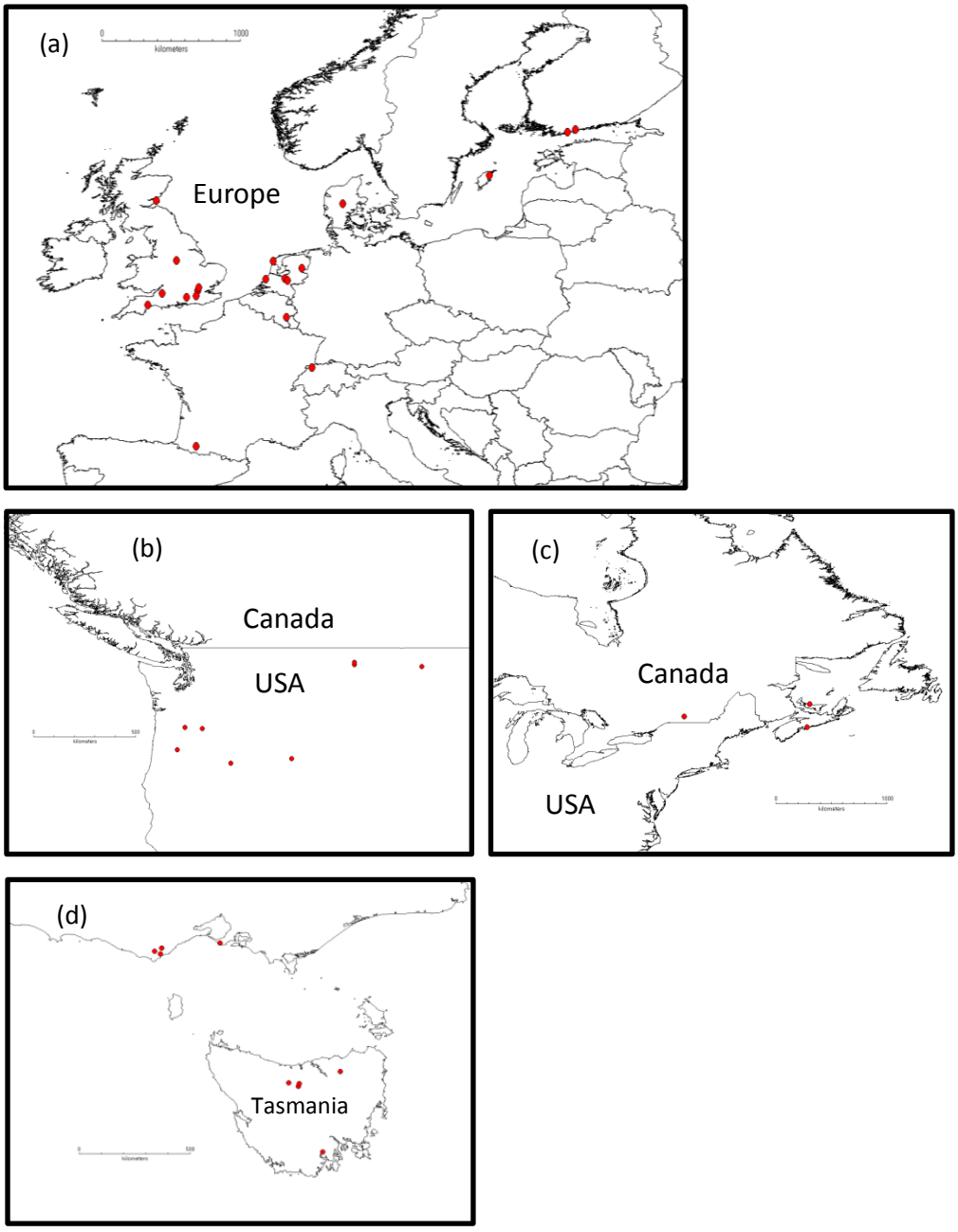
Supplementary Table S1: Origin of seeds of native and invasive populations of *Jacobaea vulgaris* used in this study.

Origin	***Country***	***Location***	***Longitude***	***Latitude***
Invasive	Australia	Dairy Plains, Tasmania	146°31' E	41°38' S
		Targa,Tasmania	147°23' E	41°18' S
		Franklin,Tasmania	147°01' E	43°04' S
		Franklin,Tasmania	146°19' E	41°33' S
		Dairy Plains, Tasmania	146°32' E	41°34' S
		Wild Dog Road, Victoria	143°40' E	38°41' S
		Beech Forest, Victoria	143°33' E	38°38' S
		Barramunga, Victoria	143°41' E	38°34' S
		Cape Schanck, Victoria	144°54' E	38°27' S
	Canada	Green Cables	63°34' W	44°37' N
		Marco polo, Montreal	73°33' W	45°30' N
		Cavendish Beach	63°22' W	46°29' N
	USA	Little Wolf, Montana	115°00' W	48°21' N
		Cockran Creek, Oregon	122°58' W	44°25' N
		Indian Creek, Oregon	117°49' W	44°01' N
		West Crestmont, Oregon	121°51' W	45°22' N
		Island lake, Oregon	122°37' W	45°25' N
		Spur Road, Conrad, Montana	111°58' W	48°09' N
		No Bear Road, Oregon	120°33' W	43°48' N
		Sunrise hill, Montana	115°00' W	48°15'N
Native	Belgium	Bertogne	05°40' E	50°05' N
	Denmark	Engesvang	09°21' E	56°10' N
	Finland	Masala, Kirkkonummi	24°31' E	60°09' N
		Inkoo, Kirkkonummi	24°00' E	60°02' N
	France	Lourdes	00°13' W	43°09' N
	The Netherlands	Meijendel	4°20' E	52°07' N
		Gees, Drenthe	6°41' E	52°44' N
		Texel	4°48' E	53°05' N
		Wageningen	05°34' E	52°10' N
		Mossel	05°45' E	52°03' N
	Sweden	Gotland	18°54' E	57°43' N
	Switzerland	Mettembert	07°19' E	47°23' N
	UK	London	00°07' W	51°30' N
		Saint Andrews	02°47' W	56°20' N

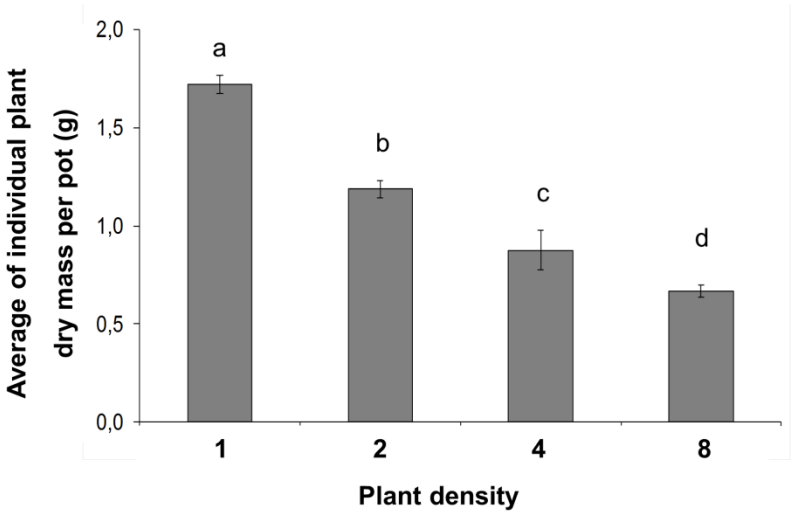
UK	Derbyshire	01°30' W	53°07' N
	Sevenoaks weald	00°12' W	51°14' N
	Marshgreen	03°21' W	50°44' N
	Enfield	00°03' W	51°40' N
	Corston	02°26' W	51°23' N
	Alice holt forest	00°50' W	51°10' N

Supplementary Table S2 Summary of ANOVAs of the difference in shoot dry mass between invasive and native genotypes from the same pot in all the competition treatments and in both harvests. The differences in shoot dry mass were tested with an ANOVA, with native population as a fixed factor. **P<0.01, ***P<0.001.

		***Tyria jacobaeae* treatment**				***Mamestra brassicae* treatment**				**No-herbivory competition control**			
		1st harvest		***2nd harvest***		***1st harvest***		***2nd harvest***		***1st harvest***		***2nd harvest***	
Source	***df***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***	***MS***	***F***
Intercept	1	0.327	1.438	8.460	1.963	23.751	75.977 ***	25.807	19.375**	12.568	50.224 ***	114.016	14.575**
Native population	19	0.205	0.903	1.884	0.437	0.544	1.795	2.744	2.060	0.523	2.090	10.211	1.305



Supplementary Figure S1. Distribution map of native and invasive Jacobaea vulgaris populations from (a) Europe n=20, (b) Western North America n=8, (c) Eastern North America n=3 and (d) Australia n=9 used in this study.



Supplementary Figure S2. Average plant total dry mass per pot from the pilot experiment at different plant densities. *Jacobaea vulgaris* plants were grown in a 0.5 L pot with 1:9 v/v potting soil and sandy soil for six weeks under same condition as in the main experiment. Different letters indicate significant differences in average plant weight among treatments at $p<0.05$ according to Post Hoc (LSD) test.

Chapter 6

Summary

For the last hundreds of years, with an increase in human travel intensity, a large amount of plant species have been introduced into new environments around the world by human activities (Cassey et al. 2005). These non-native plant species that successfully establish and spread when introduced beyond their native range and become extraordinarily prominent in their new habitats and are defined as invasive plant species (Williamson 1996; Pysek et al. 2004). The introductions of organisms beyond the natural range of potential dispersal (aided by human transport) are commonly referred as biological invasions. Invasive plant species often received a pest status in their introduced areas since their invasiveness could lead to a loss of biodiversity, cause habitat degradation and disruption and pose a threat to livestock and human health (Mack et al. 2000). Therefore invasive plant species are regarded as one of the greatest threats to global biodiversity and cause enormous economic losses (Hobbs and Mooney 1998; Kark and Antonio 2002; Pimentel et al. 2005; Pejchar and Mooney 2009; Pyšek and Richardson 2010). For example common ragwort causes more than four million dollar of annual costs in Australia due to livestock losses, decreased pasture yields and increased management costs (McLaren et al. 2000).

In spite of their negative effects, invasive species provide an ideal opportunity to ecologists for studying the evolutionary changes over time scales that are not feasible in common laboratory and field studies. Biological invasions can be used as large scale experiments where selective forces have been changed for dozens of generations. Since many biotic and abiotic factors differ between the invasive and native range the evolutionary changes that promote invasiveness remain debated (Turner et al. 2014). After plant invasions, one of the most prominent changes in selective forces is the herbivore composition in the introduced ranges. After invasion, plants are freed from their native specialist herbivores (Enemy Release Hypothesis (ERH), (Elton 1958; Keane and Crawley 2002), though they are still under herbivore pressure by the local generalist herbivores and occasional local specialist herbivores of congeneric plant species (Frick 1972; Castells et al. 2013). Such a shift in the herbivore composition towards a guild that is dominated by generalist herbivores in the new range, was expected to exert altered selection in favor of invasive genotypes with reduced allocation to anti-herbivore strategies and increased allocation to growth. Therefore the invasive plants were hypothesized to have lower defense levels, grow faster and produce more seeds and thus show an increased competitive ability over the local plant species as suggested by the Evolution of Increased Competitive Ability hypothesis (EICA), (Blossey and Nötzold 1995).

However, it is worth to point out that while invasive species are freed from their specialist herbivores they still are under attack by generalist herbivores in the invasive range (Agrawal and Kotanen 2003; Siemann and Rogers 2003; Parker et al. 2006). Therefore as an extension to the Evolution of Increased Competitive Ability hypothesis, the Shifting Defense Hypothesis (SDH) takes the different selective pressures of specialist and generalist herbivores into account (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011). The SDH states that the herbivore guild shifts from a specialist dominated community towards a generalist dominated community in the introduced range and hence invasive plants are selected for lower investment in their total defense by reducing the costly quantitative defenses against specialist herbivores and increasing the cheap qualitative defenses targeted at local generalist herbivores without having the side effect of attracting the specialist herbivores (Feeny 1976; Rhoades and Cates 1976; van der Meijden 1996). As a result a net gain can be saved for additional growth and resulting in an increased competitive ability.

Quantitative defenses are digestibility reducers and/or structural defenses such as leaf thickness and toughness that act against both specialist and generalist herbivores. They occur in high concentrations which make them expensive for plants to produce. Qualitative defenses are toxins or deterrents (e.g. alkaloids and glucosinolates) that are effective at in low concentrations and act against generalist herbivores. Because they occur at relatively low concentrations they are cheaper to produce than quantitative defenses. The drawback of qualitative defenses is that specialist herbivores are often adapted to the them and can even use them as feeding and oviposition stimulant or even sequester them for their own defense against predators (Feeny 1976; Rhoades and Cates 1976).

Instead of deterring herbivores, plants can also reduce the negative fitness effects of herbivores by being tolerant to damage (van der Meijden et al. 1988). Tolerance is the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores. Tolerance is usually seen as a last resort against specialist herbivores which have broken through other defences (Agrawal et al. 1999; Strauss and Agrawal 1999; Fornoni 2011). It is considered to be costly because leaf tissue is lost through herbivory and reserves for tolerance cannot be used for growth (Bossdorf et al. 2004b). Regrowth ability is the most common tolerance strategy of plants and it is a compensatory response to replace damaged tissue after herbivory by using the stored nutrients and energy in plant roots that are relatively free from herbivory (McNaughton 1983; Rosenthal and Kotanen 1994; de Jong and van der Meijden 2000; Anten and Pierik 2010).

However, roots have multiple functions and they can be used by plants for maintaining structure, retrieving nutrients and water for growth and for storing resources. A large root to shoot ratio may be the result of high storage levels but can also be the consequence of low nutrient availability (van der Meijden et al. 2000). To understand regrowth capacity and the role of the size of the roots for regrowth it is necessary to study both the root storage and root size.

So far a lot of studies compared invasive plants with their conspecifics and only a few of them showed strong evidence that evolutionary changes have been occurred in invasive plant genotypes (Daehler 2003; Vila and Weiner 2004; van Kleunen et al. 2010; Palacio-Lopez and Gianoli 2011). However, even among the latter studies evidence showing that changes in the herbivore guild are the selective force for changes in allocation patterns is largely circumstantial as other biotic and/or abiotic factors cannot be ruled out (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Colomer-Ventura et al. 2015). Therefore we set out to study a system where multiple invasive regions are compared that differ in climatological conditions. If a change in the herbivore guild is the main selective force acting on growth, competitive ability and anti-herbivore defenses, parallel evolutionary changes are expected in each of the geographically and climatologically differing invasive ranges.

In this thesis I used *J. vulgaris* as s study plant species. With several experimental setups I compared the differences in growth and growth related traits, qualitative defense (pyrrolizidine alkaloids), quantitative defense (structural defenses and tolerance) and competitive ability between native and invasive *J. vulgaris* genotypes. In addition, I specifically selected the invasive genotypes from ranges that are geographically isolated and that do differ in climatological conditions. The main goal of this thesis is to exam whether there are evolutionary changes in invasive *J. vulgaris* plants and if so, whether these changes are due to the release of the selection pressure from specialist herbivores in the introduced area?

In my thesis the following four specific research questions are proposed:

1. Do invasive *Jacobaea vulgaris* genotypes have decreased quantitative defenses (structural defenses and regrowth)?
2. Do invasive *Jacobaea vulgaris* genotypes have increased qualitative defense (chemical defense)?
3. Do invasive *Jacobaea vulgaris* genotypes have increased growth and competitive ability in the absence of herbivores?
4. Is the competitive ability of invasive *Jacobaea vulgaris* genotypes more affected by a specialist herbivore and less affected by a generalist herbivore than that of native genotypes?
5. Did evolution select growth, competitive ability, quantitative and qualitative defenses of invasive *Jacobaea vulgaris* genotypes from geographically and climatological distinct regions all towards the predicted directions?

Corresponding to these research questions I will divide my discussion into five parts.

1. Evolutionary changes in growth, structural defenses and potential regrowth ability in invasive *Jacobaea vulgaris*

In chapter 2 I studied the differences in growth, structural defense and potential regrowth ability (root-shoot ratio) between native and invasive *J. vulgaris* genotypes in which plants grew in individual pots in a climate room for 17 weeks. I examined structural defense related

traits such as leaf microscopically structural traits (e.g. leaf thickness, cell wall thickness), toughness of leaves, amount of cell wall proteins in leaves, and root-shoot ratio. In addition, plant biomass at the end of the experiment was measured. Following the EICA and SDH I hypothesized that due to the absence of specialist herbivores but the presence of generalist herbivores, invasive *J. vulgaris* genotypes have been selected for decreased structural defenses and regrowth but increased growth performance.

In this study I did not find strong evidence from microscopic analysis and leaf toughness analysis supporting the hypothesis that invasive genotypes have evolved lower structural defenses against herbivory. But I did find that invasive *J. vulgaris* had 7% thinner leaves (results are summarized in Table 1). Leaf thickness plays an important role in plant anti-herbivore defense. Peeters (2002) found it was negatively associated with densities of chewing herbivores. In addition, I found that the invasive *J. vulgaris* contained a 10.8% lower amount of cell wall proteins per leaf area than the native ones (Table 1). Leaf cell walls can constitute a substantial amount of nitrogen and account for 30-50% of leaf dry mass. Therefore having a smaller amount of cell walls can decrease leaf structural defense which, in turn, would render plants to be more susceptible to herbivores (Onoda et al. 2004). The results further showed that invasive *J. vulgaris* genotypes invested more in the aboveground parts than in underground parts resulting in a 13.7% larger shoot mass and a 8% smaller leaf mass area. Having larger shoots and thinner leaves may enable invasive genotypes to grow faster (Lake and Leishman 2004; Leishman and Thomson 2005; Grotkopp and Rejmánek 2007), and, independent of this growth potential, also enable them to compete more effectively for light (Schieving and Poorter 1999). Therefore plants from the invasive *J. vulgaris* genotypes were expected to have higher potential growth than native plants. At the final harvest we found no differences in total dry mass because invasive plants produced smaller roots. As a result, the invasive plants had a 18.7% lower root-shoot ratio. A low root-shoot ratio in the invasive plants can represent poorer potential regrowth ability. This is supported by the findings of Joshi and Vrieling (2005) who found that native *J. vulgaris* genotypes had a 12% higher regrowth ability after complete defoliation. In general, this study is consistent with the EICA which hypothesize that invasive *J. vulgaris* have evolved poorer structural defenses and lower tolerance ability to herbivory but a higher potential growth compared to native genotypes.

2. Evolutionary changes in growth, chemical defense and potential regrowth ability in invasive and native *Jacobaea vulgaris*

In chapter 3 I studied the differences in growth, chemical defense, and regrowth ability between native and invasive *J. vulgaris* genotypes. I grew plants individually in pots in a climate room for 9 weeks before harvest. After that I examined plant growth and underlying traits such as plant biomass, photosynthesis, specific leaf area and leaf mass fraction. In addition, the quantity and composition of leaf pyrrolizidine alkaloids (PAs) in each plant was measured by LC-MS as a measure of qualitative defense. Root carbohydrate storage (inulin) was measured as the energy source for regrowth. Following the EICA and SDH I hypothesized that due to the absence of specialist herbivores but the presence of generalist herbivores, invasive *J. vulgaris* genotypes have been selected for decreased regrowth ability but increased growth and qualitative chemical defense PA concentration.

In this study I found that invasive *J. vulgaris* had 5% higher specific leaf area, 12% higher leaf mass fraction, 7.7% higher Pmax and a 10.8% higher PNUE than native genotypes (Table 1). All these underlying growth traits are assumed to be positively correlated with relative growth rate (Poorter and Remkes 1990; Shipley 2006). Indeed at the end of this experiment invasive *J. vulgaris* produced a 12% larger total dry mass. This is in line with a previous study (Joshi and Vrieling 2005) who found that invasive *J. vulgaris* plants had higher vegetative growth and had a 37% higher reproductive output compared to native plants in a common garden experiment after eight months of growth. However, this result is not in line with Willis et al. (2000). The latter found no difference in size between invasive and native *J. vulgaris* which is may be due to the small sample size that has been used in this study (6 populations for each range).

The data further showed that invasive *J. vulgaris* produced on average a 43% higher concentration of total PAs and 123 % higher concentration of tertiary amine PAs than native genotypes while the concentration of N-oxide PAs was similar (Table 1). When we further compared the invasive *J. vulgaris* genotypes with the potential source populations from the western coast of Europe (Doorduin et al. 2010), they did not differ from native genotypes in total PA concentration but their PA composition had shifted to the more toxic jacobine-like PAs and only trace amount of Erucifoline-like PAs. Jacobine-like PAs are present in larger quantities as tertiary amines. These findings are in line with Joshi & Vrieling (2005). Since tertiary amines were observed to be more deterrent to insects than the PA N-oxides (Macel et al. 2005; Nuringtyas et al. 2014), this indicates that invasive genotypes have shifted to more toxic forms of PAs than native genotypes. Since generalist herbivores play a role in the evolution and maintenance of the diversity of PAs (Macel et al. 2005), I argue that the changes in the herbivore guild towards one dominant by generalists in the introduced areas has led to selection for increased qualitative defense in invasive *J. vulgaris*.

Invasive *J. vulgaris* stored 34% less carbohydrate (inulin) in the roots compared to native genotypes (Table 1). As one of the underlying traits of regrowth, root carbohydrate storage was found to be positively correlated with regrowth ability in several plant species (Donaghyē and Fulkersonǵ 1998; Sosnová and Klimešová 2009; Chen et al. 2013; McCormick et al. 2013; Aranjuelo et al. 2015). Also Joshi and Vrieling (2005) showed, by clipping in a in a common garden experiment, that native *J. vulgaris* genotypes had better regrowth after complete defoliation. This study clearly showed that evolutionary changes in the allocation patterns of invasive *J. vulgaris* populations have occurred and they have evolved less root storage for potential regrowth but increased growth and qualitative chemical defense (PAs) after invasion as predicted by the SDH hypothesis.

3. Evolutionary changes in growth and regrowth ability of invasive *Jacobaea vulgaris*

In the chapter 2 and 3 we found the invasive *J. vulgaris* have evolved poorer underlying regrowth traits such as smaller root-shoot ratio and less root carbohydrate storage. In order to test the actual regrowth ability, I conducted an experiment (chapter 4) with artificial defoliation to measure the effects on growth and regrowth in invasive and native *J. vulgaris*. In this experiment, I examined plant growth and root carbohydrate storage (inulin content) before defoliation and after four weeks of regrowth.

The results showed that invasive *J. vulgaris* genotypes had better growth by producing 17% higher total mass and a 44% larger total leaf area a than native genotypes in the control treatment without defoliation after 12 weeks of growth (Table 1). In contrast, the invasive *J. vulgaris* from the clipping treatment showed a poorer regrowth resulting in a 20% lower total leaf area and a 20% lower total dry mass than their native congeners after four weeks of regrowth. Plants from invasive *J. vulgaris* genotypes contain a 38% lower of root inulin content than that of native genotypes at the moment of clipping and I found that the amount of root inulin content is positively correlated with the net gain in total biomass after four weeks of regrowth. Similarly, root carbohydrate storage has been reported to play an important role in regrowth after defoliation in several plant species (Ta et al. 1990; Corre et al. 1996; Avice et al. 1997; McCormick et al. 2013; Janeček and Klimešová 2014). The results strongly suggest that invasive *J. vulgaris* have been selected to compromise their regrowth ability to increase plant growth through a lower investment in root carbohydrate storage. The data further suggest that a large root is not necessarily translated into better regrowth ability. In fact the size of structural root was negatively correlated with plant regrowth after damage. Therefore it is crucial to study both the root storage and root size in order to understand plant regrowth ability and the role of the size of the roots for regrowth.

4. Evolutionary changes in competitive ability and regrowth in invasive and native *Jacobaea vulgaris* with and without herbivory

Generally a higher growth rate is considered as a proxy for competitive ability (Grime 2006). Since plant performance in the absence of competition might not necessarily translate into competitive ability, it is essential to examine plant competitive ability in a competitive environment (Bossdorf et al. 2004a). The outcome of competition experiments between native and invasive plants is also likely to depend strongly on whether or not herbivory is included and which herbivores are present since the invasive and native genotypes have been selected to evolve different anti-herbivore strategies. Therefore, in chapter 5, I compared the competitive ability of invasive and native *J. vulgaris* genotypes with an intraspecific competition setup where native and invasive genotypes compete with each other. The mixed cultures were exposed to three treatments: no herbivores, a generalist herbivore (*Mamestra brassicae*) or a specialist herbivore (*Tyria jacobaeae*). I harvested half of the pots after 12 days of herbivory and from the remaining pots the herbivores were removed and the plants were allowed to (re)grow for another four weeks. Shoot biomass of individual plants was measured at both harvests.

The results showed that invasive *J. vulgaris* genotypes were less preferred by the generalist *Mamestra brassicae* but more preferred by the specialist *Tyria jacobaeae*. Consequently the competitive ability of invasive genotypes was significantly increased by the first and reduced by the latter (Table 1). The increased competitive ability of the invasive genotypes was accompanied by a decrease in regrowth ability after attack by either type of herbivores. The latter result is in line with the results of the previous chapters. All together my results are in line with the EICA and SDH hypotheses and show that herbivore species can play a decisive role in shaping the competitive ability of invasive plants. The shift in the herbivore guild in the introduced range towards a community dominated by generalists has selected for invasive plants with better competitive ability in the presence of generalist herbivores. This

most likely contributed to their invasion success. More importantly, my results indicate that a competition experiment without generalist herbivores underestimates the competitive ability of plants from the invasive range.

Besides this thesis, I did some preliminary experiments about the preference of other generalist herbivores (slugs, leaf miners, and thrips) using *J. vulgaris* leaf discs. I found that slugs and leaf miners preferred invasive genotypes while thrips preferred native ones. Although at the first sight this is not in line with our hypothesis, it should be noted that damage by both slugs and leaf miners did not show a negative correlation with Jacobine concentration while damage by thrips did show such a negative correlation (Wei et al. 2015). An increase in Jacobine concentration was the most prominent evolutionary change in qualitative defense in invasive *Jacobaea vulgaris* genotypes. In addition, these results may be explained by differences in feeding behaviors among herbivore species. Accordingly, selection forces plants to co-evolve relevant strategies by distributing their defensive metabolites to different locations (Martin et al. 2001). Indeed, plant metabolites have been reported to be highly diversely distributed in different leaf tissues such as epidermis, cell walls, vacuoles, and cell nuclei (Hrazdina et al. 1982; Hutzler et al. 1998; Nuringtyas et al. 2012). Therefore the conclusion from chapter 5 should be treated with some cautions. More generalist herbivore species should be further tested with this study system and the differences in defensive metabolites between native and invasive *J. vulgaris* genotypes among different leaf tissues should be studied in more detail as well.

5. Parallel evolution in different invasive regions

In this study we found that of the traits measured (growth, competitive ability, anti-herbivore defenses and regrowth ability) which are significantly different between native and invasive genotypes of *J. vulgaris*, the invasive *J. vulgaris* populations from different geographically distinct regions all changed consistently towards the predicted direction. Combined with the fact that the local climate conditions differed significantly among the invasive regions, our result strongly suggests that the parallel evolution have occurred among the invasive *J. vulgaris* populations from geographically distinct regions in response to the shift in the herbivore pressure. Besides that, it is worth to point out that alternative explanations for parallel evolution e.g. bridgehead or anthropogenically induced adaptation to invasive regions are less likely to play a role (Lombaert et al. 2010; Hufbauer et al. 2012). Since genetic analyses of native and invasive *J. vulgaris* showed that multiple introductions have occurred in the invasive ranges (Doorduin et al. 2010), suggesting that the changes in the traits can be best explained by a parallel evolution in the invasive *J. vulgaris* populations from the different introduced regions.

Synthesis

This thesis shows that natural selection within less than 70 generations changed allocation patterns of invasive *J. vulgaris* genotypes to evolve better growth and competitive ability as well as higher qualitative defense while the investment in structural defenses and regrowth ability are reduced (Table 1). These results support the Evolution of Increased Competitive Ability hypothesis and the Shifting defense hypothesis. Several other invasive plant species

evolved the similar changes in the allocation patters as *J. vulgaris* (Liu and Stiling 2006; Doorduin and Vrieling 2011; Felker-Quinn et al. 2013). However, evidence collected so far was still insufficient to prove that the changes in the herbivore guilds are the responsible selective force because other biotic and/or abiotic factors cannot be ruled out as being important (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Colomer-Ventura et al. 2015). Therefore this thesis is one of the first studies that have compared native populations with invasive populations of the same plant species from multiple invasive regions which are geographically and climatologically distinct. Using such a setup, the other abiotic factors can be possibly ruled out since the climate conditions are considered as the most potential abiotic selective force for post-invasive evolution (Bradley et al. 2009; Colomer-Ventura et al. 2015). My results showed that all the studied traits changed consistently in the same predicted direction in all invasive *J. vulgaris* populations from multiple regions. This strongly suggests that the absence of specialist herbivores was the selective force leading to these parallel evolutionary changes in invasive *J. vulgaris*. It remains to be seen if the same holds for other invasive plant species. Clearly more species should be studied using a similar set-up to evaluate the role of herbivores in the invasiveness of plant species. Understanding the selective forces leading to evolutionary changes in invasive species can help to control invasive pests.

Table 1 Summary of the differences in the main traits between native and invasive *Jacobaea vulgaris* populations. Values are Mean ± SE, P values are from a nested ANOVA, with origin (native versus invasive) as fixed factor, invasive regions nested within origin and populations nested within invasive regions as random factors. P (origins): significance level of nested ANOVA between invasive and native origins. NS= not significant.

Chapter #	***Traits***	***Invasive***	***Native***	***P (Origins)***
Chapter 2	Leaf thickness (µm)	264.53±3.08	276.62±2.95	0.023
(17weeks of growth)	Leaf toughness (kJ•m^{-2})	0.227±0.009	0.210±0.007	NS
	Leaf mass area (g•m^{-2})	57.55±1.50	62.74±1.88	0.038
	Cell wall proteins (g•m^{-2})	9.48±0.31	10.63±0.25	0.037
	Total dry mass(g)	11.74±0.52	12.20±0.49	NS
	Root-shoot ratio (g•g^{-1})	0.750±0.037	0.922±0.046	0.030
Chapter 3	Total dry mass (g)	3.73±0.14	3.33±0.11	0.041
(9 weeks of growth)	Specific leaf area (cm^2•g^{-1})	238.09±2.14	228.3±2.48	0.044
	Leaf mass fraction (g•g^{-1})	0.728±0.005	0.649±0.006	<0.001
	Pmax (µmol CO_2•m^{-2}•s^{-1})	23.13±0.43	21.48±0.38	0.005
	PNUE(µmol CO_2•g^{-1}•s^{-1})	18.86±0.44	17.02±0.41	0.011
	Total PA (µg•g^{-1} DW)	4144.6±152.8	2893.3±162.6	0.001
	Total PA (Tertiary amines) (µg•g^{-1} DW)	1954.9±90.02	876.90±98.28	<0.001
	Total PA (N-oxide) (µg•g^{-1} DW)	2189.7±114.4	2016.4±104.8	NS
	Total inulin content in root (g)	0,397±0,025	0,597±0,028	<0.001
Chapter 4	Total dry mass at the moment of clipping (g)	2,14±0,09	1,90±0,08	NS (0,061)
(8 weeks of growth and 4 weeks of regrowth)	Total dry mass in the control treatment (g)	8,69±0,27	7,45±0,32	0,01
	Total dry mass after 4 weeks of regrowth (g)	1,58±0,06	1,97±0,09	0,003
	root inulin content at the moment of clipping (g)	0,209±19,84	0,339±22,64	<0,001
Chapter 5	Shoot dry mass (g)			
(8 weeks of growth, 12 days of herbivory and 4 weeks of regrowth)	After specialist herbivory	1.31±0.07	1.41±0.09	NS
	After generalist herbivory	2.18±0.10	1.23±0.07	<0.001
	Without herbivore	2.34±0.09	1.63±0.08	<0.001
	Shoot dry mass after regrowth (g)			
	After specialist herbivory	1.90±0.20	2.49±0.15	0.037
	After generalist herbivory	3.22±0.26	2.27±0.18	0.010
	Without herbivore	5.16±0.42	3.31±0.28	0.002

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Nederlandse samenvatting

(Dutch summary)

Invasieve plantensoorten worden gedefinieerd als niet-inheemse plantensoorten die zich succesvol vestigen en verspreiden buiten hun inheemse verspreidingsgebied en in hun nieuwe habitat buitengewoon prominent aanwezig zijn (Williamson 1996; Pysek et al. 2004). In het gebied waar ze geïntroduceerd zijn kunnen ze een verlies aan biodiversiteit, habitat degradatie en verstoring veroorzaken en een bedreiging vormen voor de gezondheid van mensen en vee (Hobbs and Mooney 1998; Kark and Antonio 2002; Pimentel et al. 2005; Pejchar and Mooney 2009; Pyšek and Richardson 2010).

Ondanks de negatieve effecten, bieden invasieve soorten ecologen een ideale mogelijkheid om evolutionaire veranderingen te bestuderen door invasies als grootschalige experimenten te beschouwen waarbij gedurende tientallen generaties grote veranderingen in selectieve krachten hebben plaatsgevonden. Aangezien veel biotische en abiotische factoren verschillen tussen het invasieve en inheemse verspreidingsgebied blijft het echter onduidelijk welke evolutionaire veranderingen invasiviteit bevorderen (Turner et al., 2014). Na de invasie van een plant is een van de meest prominente veranderingen in selectieve krachten de herbivoor samenstelling in de geïntroduceerde gebieden. Planten worden bevrijd van hun inheemse specialistische herbivoren“Enemy Release Hypothese” (ERH), (Elton 1958, Keane en Crawley 2002), hoewel ze nog steeds de herbivorendruk ondervinden van de lokale generalistische herbivoren (Frick 1972) en incidentele lokale specialistische herbivoren van verwante plantensoorten (Castells et al 2013.). Een dergelijke verschuiving in de herbivorensamenstelling in het nieuwe gebied naar een gemeenschap die wordt gedomineerd door generalistische herbivoren, zou een andere selectiedruk uitoefenen die zou leiden tot een verlaagde allocatie naar anti-herbivoor strategieën en verhoogde allocatie naar groei bij invasieve genotypen. De “Evolution of Increased Competitive Ability” (EICA) hypothese stelt daarom dat invasieve planten een lager afweerniveau hebben, sneller groeien en meer zaden produceren en hierdoor en verhoogd competitief vermogen hebben ten opzichte van lokale plantensoorten als gevolg van evolutionaire veranderingen (Blossey and Nötzold 1995).

Het is belangrijk te weten dat hoewel invasieve soorten in hun invasieve gebied bevrijd zijn van hun specialistische herbivoren, zij nog steeds aangevallen worden door generalistische herbivoren (Agrawal and Kotanen 2003; Siemann and Rogers 2003; Parker et al. 2006). Als uitbreiding van de EICA hypothese, houdt de “Shifting Defense Hypothesis” (SDH) rekening met de verschillende selectiedrukken van specialistische en generalistische herbivoren (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduin and Vrieling 2011). De SDH stelt dat in het geïntroduceerde gebied de samenstelling van de herbivorengemeenschap verschuift van een door specialisten gedomineerde gemeenschap naar een door generalisten gedomineerde gemeenschap waardoor invasieve planten worden geselecteerd die minder investeren in hun algehele afweer door hun dure kwantitatieve afweer tegen specialisten te verlagen en hun goedkope kwalitatieve afweer tegen lokale generalisten te verhogen zonder de bijwerking dat de specialistische herbivoren worden aangetrokken (Feeny 1976; Rhoades and Cates 1976; van der Meijden 1996). Als resultaat kan een netto

winst worden gebruikt voor additionele groei, resulterend in een toename van het competitieve vermogen.

Tot nu toe hebben veel studies invasieve planten met hun verwante plantensoorten vergeleken en enkele daarvan voerden sterk bewijs aan dat evolutionaire veranderingen hadden plaats gevonden in de invasieve genotypen (Daehler 2003; Vila and Weiner 2004; Van Kleunen et al. 2010; Palacio-Lopez and Gianoli 2011). Zelfs in deze laatste studies is het bewijs dat aantoont dat veranderingen in de herbivoren gemeenschap de selectieve kracht is voor veranderingen in allocatiepatroon is echter voornamelijk indirect en het kan niet uitgesloten worden dat andere biotische of abiotische factoren ook een rol spelen (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Colomer-Ventura et al. 2015).

Om deze reden gebruiken wij in dit proefschrift een opzet waarbij meerdere invasieve gebieden die verschillen in klimatologische condities worden vergeleken. Als een verandering in herbivoren gemeenschap de belangrijkste selectieve kracht is werkend op groei, competitievermogen en anti-herbivoren afweer, is te verwachten dat parallelle evolutionaire veranderingen plaatsvinden in elk van de geografisch en klimatologisch verschillende gebieden.

In dit proefschrift gebruikte ik de plantensoort J. vulgaris als studieobject. In verschillende experimenten vergeleek ik verschillen tussen inheemse en invasieve J. vulgaris in groei en groei gerelateerde eigenschappen, kwalitatieve afweer (pyrrolizidine alkaloïden), kwantitatieve afweer (structurele afweer en tolerantie) en concurrerend vermogen tussen inheemse en invasieve J. vulgaris. Ik selecteerde hiervoor specifiek die invasieve genotypen uit gebieden die geografisch geïsoleerd zijn en verschillen in klimatologische condities. Het hoofddoel van dit proefschrift is het onderzoeken of er evolutionaire veranderingen plaatsvinden in invasieve J. vulgaris planten en indien dit het geval is of deze het resultaat zijn van het verdwijnen van de selectiedruk van specialistische herbivoren in het geïntroduceerde gebied.

In mijn proefschrift worden de volgende vier specifieke onderzoeksvragen behandeld:

1. Hebben invasieve *Jacobaea vulgaris* genotypen verminderde kwantitatieve afweer (structurele afweer en hergroei) ten opzichte van inheemse *Jacobaea vulgaris* genotypen?
2. Hebben invasieve *Jacobaea vulgaris* genotypen toegenomen kwalitatieve afweer (chemische afweer) ten opzichte van inheemse *Jacobaea vulgaris* genotypen?
3. Hebben invasieve *Jacobaea vulgaris* genotypen toegenomen groei en competitief vermogen in de afwezigheid van herbivoren ten opzichte van inheemse *Jacobaea vulgaris* genotypen?
4. Wordt het competitief vermogen van invasieve *Jacobaea vulgaris* genotypen meer beïnvloed door een specialistische herbivoor en minder beïnvloed door een generalistische herbivoor?
5. Heeft de evolutie groei, competitief vermogen, kwantitatieve en kwalitatieve afweer van invasieve *Jacobaea vulgaris* van geografische en klimatologische gescheiden gebieden allemaal in de verwachte richtingen geselecteerd?

Corresponderend met deze onderzoeksvragen heb ik mijn discussie verdeeld in vijf delen.

1. Evolutionaire veranderingen in groei, structurele afweer en potentiele hergroei capaciteit in invasieve *Jacobaea vulgaris*

In hoofdstuk twee bestudeerde ik de verschillen in groei, structurele afweer en potentiele hergroei capaciteit (wortel-spruit verhouding) tussen inheemse en invasieve *J. vulgaris* genotypen. Planten groeiden in individuele potten in een klimaatkamer gedurende 17 weken. Ik bestudeerde eigenschappen gerelateerd aan structurele afweer zoals bladdikte, celwanddikte, stugheid van de bladeren, hoeveelheid celwandeiwitten in de bladeren, en wortel-spruit verhouding. Bovendien werd ook de planten biomassa gemeten aan het eind van het experiment.

In dit onderzoek vond ik voor bladdikte, celwanddikte en blad stugheid geen sterk bewijs dat de hypothese ondersteund dat invasieve genotypen een lagere structurele afweer tegen herbivorie hebben geëvolueerd. Ik vond wel dat invasieve *J. vulgaris* 7% dunnere bladeren hadden dan inheemse *J. vulgaris* genotypen (Tabel 1). Bladdikte speelt een belangrijke rol in herbivorenafweer. Bovendien vond ik dat invasieve *J. vulgaris* 10.8% lagere hoeveelheden celwandeiwitten per bladoppervlakte bevatte in vergelijking met inheemse *J. vulgaris* (Tabel 1). De resultaten toonden verder dat invasieve *J. vulgaris* genotypen meer investeren in bovengrondse delen dan in ondergrondse delen wat resulteert in een 13.7% grotere spruit massa en een 8% kleinere bladmassa per oppervlakte-eenheid. Het hebben van grotere spruiten en dunnere bladeren kan invasieve genotypen de mogelijkheid geven om sneller te groeien (Lake and Leishman 2004; Leishman and Thomson 2005; Grotkopp and Rejmánek 2007), en onafhankelijk van dit groei potentieel, biedt dit ze de mogelijkheid om effectiever te concurreren om licht (Schieving and Poorter 1999). Verwacht werd daarom dat planten van het invasieve *J. vulgaris* genotype een hogere potentiële groei hebben dan inheemse planten. Bij de laatste oogst vonden we geen verschil in drooggewicht doordat invasieve planten kleinere wortels hadden. Als resultaat hiervan hadden invasieve planten een 18.7% lagere wortel-spruit verhouding. Een lage wortel-spruit verhouding in de invasieve planten zou kunnen duiden op een slechtere potentiele hergroei. In het algemeen zijn de resultaten van dit onderzoek consistent met de “EICA” en “SDH” hypothese die stelt dat in invasieve *J. vulgaris* in vergelijking met inheemse genotypen slechtere structurele afweer en lagere tolerantie voor herbivorie, maar een groter potentiele groei geëvolueerd is.

2. Parallelle evolutionaire veranderingen in groei, chemische afweer en potentiele hergroei capaciteit in invasieve en inheemse *Jacobaea vulgaris* planten

In hoofdstuk 3 bestudeerde ik de verschillen in groei, chemische afweer en hergroei capaciteit tussen inheemse en invasieve *J. vulgaris* genotypen. Planten groeiden in individuele potten in een klimaatkamer gedurende 9 weken tot ze geoogst werden. Hierna bestudeerde ik plantengroei en onderliggende eigenschappen zoals plant biomassa, fotosynthese, specifiek bladoppervlak en de fractie bladmassa. Hiernaast werd als een maat van kwalitatieve afweer in elke plant de kwantiteit en samenstelling van pyrrolizidine alkaloïden (PA’s) in het blad gemeten met behulp van LC-MS. De opslag van koolwaterstoffen (inuline) in de wortels werd gemeten als de energiebron voor hergroei.

In dit onderzoek vond ik dat invasieve *J. vulgaris* een 5% grotere specifiek bladoppervlakte, een 12% grotere fractie bladmassa, een 7.7% grotere Pmax en een 10.8% grotere PNEU hadden in vergelijking met inheemse genotypen (Tabel 1). Al deze eigenschappen worden aangenomen positief gecorreleerd te zijn met relatieve groei (Poorter and Remkes 1990; Shipley 2006). Aan het eind van het experiment produceerde invasieve *J. vulgaris* inderdaad een 12% groter drooggewicht. De data liet verder zien dat invasieve *J. vulgaris* gemiddeld een 43% hogere concentratie totale PA's en 123% hogere concentratie tertiaire amine PA's produceerde in vergelijking met inheemse genotypen terwijl de concentratie N-oxide PA's gelijk was (Tabel 1). Toen we de invasieve *J. vulgaris* genotypen verder vergeleken met de potentiële bron populaties van de westelijke kust van Europa (Doorduin et al. 2010) vonden we geen verschil met de inheemse genotypen in totale PA concentratie maar de PA samenstelling was verschoven naar meer toxische jacobine-achtige alkaloïden. Deze bevindingen komen overeen met Joshi & Vrieling (2005). Aangezien waargenomen werd dat tertiaire amines meer afschrikwekkend tegen insecten zijn dan PA N-oxides (Macel et al. 2005; Nuringtyas et al. 2014) impliceert dit dat invasieve genotypen meer toxische vormen van PA's produceren in vergelijking met inheemse genotypen. Bovendien sloeg *J. vulgaris* 34% minder reservestoffen (inuline) op in de wortels in vergelijking met de inheemse genotypen (Tabel 1). Voor alle bestudeerde eigenschappen gold dat deze op gelijke wijze in de bestudeerde invasieve gebieden veranderden ondanks dat de klimatologische omstandigheden in deze gebieden duidelijk van elkaar verschilden.

Dit onderzoek toonde duidelijk dat er evolutionaire veranderingen in de allocatie patronen van invasieve *J. vulgaris* populaties hebben plaatsgevonden en dat deze na deze evolutionaire veranderingen na invasie minder opslag in de wortels voor potentiele hergroei hebben maar toegenomen groei en kwalitatieve afweer (PA's) zoals voorspelt door de "SDH" hypothese.

3. Parallelle evolutionaire veranderingen in groei en hergroei capaciteit van invasieve *Jacobaea vulgaris*

In hoofdstuk 4 voerde ik een experiment uit waarin ik planten kunstmatig ontbladerde om de effecten hiervan op groei en hergroei in invasieve en inheemse *J. vulgaris* te meten. In dit experiment, mat ik voor ontbladering en 4 weken na hergroei, de plantengroei en de opslag van koolwaterstoffen (inuline) in de wortels.

De resultaten toonden aan dat na 12 weken hergroei invasieve *J. vulgaris* genotypen een betere hergroei hadden met een productie van 17% meer massa en 44% groter totaal bladoppervlak in vergelijking met inheemse genotypen in de controlebehandeling zonder ontbladering (Tabel 1). Dit in tegenstelling tot de invasieve *J. vulgaris* van de ontbladerde behandeling die na vier weken hergroei een 20% kleiner totale bladoppervlak en een 20% lagere totale droge massa vertoonden in vergelijking met inheemse genotypen. Op het moment van ontbladering bevatten de wortels van planten van het invasieve *J. vulgaris* genotype 38% minder inuline in vergelijking met wortels van inheemse genotypen. Ik vond dat de hoeveelheid inuline in de wortels positief gecorreleerd was met de netto toename van de totale biomassa na vier weken hergroei. Ander onderzoek beschreef eerder dat de opslag van reservestoffen in wortels een belangrijke rol speelt bij hergroei na ontbladering

in verschillende plantensoorten (Ta et al. 1990; Corre et al. 1996; Avice et al. 1997; McCormick et al. 2013; Janeček and Klimešová 2014). De resultaten wijzen er sterk op dat in invasieve *J. vulgaris* selectie heeft plaatsgevonden naar een lagere hergroei capaciteit en een toegenomen plantengroei door minder investering in de opslag van reservestoffen in wortels. De data lijkt er verder op te wijzen dat grote wortels niet noodzakelijkerwijs betekenen dat er sprake is van een betere hergroei capaciteit aangezien de grootte van de wortel zonder reserve stoffen negatief gecorreleerd was met hergroei na ontbladering. Het is daarom cruciaal om zowel de opslag in de wortels als de wortelgrootte te bestuderen om de hergroei capaciteit van planten en de rol die de wortelgrootte hierin speelt te begrijpen. Voor alle bestudeerde eigenschappen gold dat deze op gelijke wijze in de bestudeerde invasieve gebieden veranderden ondanks dat de klimatologische omstandigheden in deze gebieden duidelijk van elkaar verschilden.

4. Paralelle evolutionaire veranderingen in concurrerend vermogen en hergroei van invasieve en inheemse *Jacobaea vulgaris* in aan- en afwezigheid van herbivoren

In hoofdstuk 5 vergeleek ik het competitief vermogen van invasieve en inheemse *J. vulgaris* genotypen in een opstelling waarin inheemse en invasieve genotype met elkaar in concurrentie groeiden. De planten werden blootgesteld aan drie behandelingen: geen herbivoren, een generalistische herbivoor (Koolmot, Mamestra brassicae) of een specialistische herbivoor (Jacobsvlinder, Tyria jacobaeae). Na twaalf dagen herbivorie oogstte ik de helft van de potten en werden de herbivoren van de overige planten verwijderd waarna deze nog vier weken konden (her)groeien. Bij beide oogsten werd de spruit biomassa van individuele planten gemeten en het wortelgewicht in de hele pot.

De resultaten toonden aan dat de invasieve *J. vulgaris* genotypen minder aantrekkelijk waren voor de generalist M. brassicae maar werden geprefereerd door de specialist T. jacobaeae. Het concurrerend vermogen van de invasieve genotypen was derhalve significant toegenomen als er generalistische herbivoren aanwezig zijn en significant afgenomen als er specialistische herbivoren aanwezig zijn (Tabel 1). Het toegenomen concurrerend vermogen van de invasieve genotypen ging hand in hand met een afname van de hergroei capaciteit na aantasting door elk van de herbivoren. Dit resultaat bevestigd de resultaten in eerdere hoofdstukken. De gevonden resultaten zijn in overeenstemming met de verwachtingen van de EICA en SDH hypotheses en tonen aan dat herbivoren een beslissende rol kunnen spelen in de vorming van het concurrerend vermogen van invasieve planten. Bovendien wijzen mijn resultaten erop dat een concurrentie experiment zonder generalistische herbivoren het concurrerend vermogen van planten uit het invasieve gebied onderschat. Ook voor het concurrerend vermogen gold dat deze in alle bestudeerde invasieve gebieden groter was dan in de inheems gebieden ondanks dat de klimatologische omstandigheden in deze gebieden duidelijk van elkaar verschilden.

5. Parallelle evolutie in verschillende invasieve gebieden

In dit onderzoek vonden we dat de gemeten eigenschappen (groei, competitief vermogen, herbivoren afweer en hergroei capaciteit) die significant verschillen tussen inheemse en invasieve *J. vulgaris* genotypen allen consistent in de verwachte richting veranderen in de

invasieve *J. vulgaris* populaties van verschillende geografisch gescheiden gebieden. Gecombineerd met het feit dat de lokale klimaatcondities significant verschilden tussen de invasieve gebieden, wijzen mijn resultaten erop dat de parallelle evolutie in de invasieve *J. vulgaris* populaties uit geografisch gescheiden gebieden heeft plaats gevonden als reactie op de verandering in herbivoren druk. Het is niet waarschijnlijk dat alternatieve verklaringen voor parallelle evolutie zoals het 'bridgehead' effect of antropogeen geïnduceerde adaptatie in invasieve gebieden een rol spelen (Lombaert et al. 2010; Hufbauer et al. 2012). Aangezien genetische analyses van inheemse en invasieve *J. vulgaris* aantoonde dat er meerdere introducties hebben plaatsgevonden in het invasieve gebied (Doorduin et al. 2010), kunnen de veranderingen in eigenschappen het best verklaard worden door parallelle evolutie in de invasieve populaties uit de verschillende geïntroduceerde gebieden.

Synthesis

Dit proefschrift toont aan dat natuurlijke selectie in minder dan 70 generaties de allocatiepatronen van invasieve *J. vulgaris* genotypen verandert heeft waardoor deze zowel betere groei en concurrerend vermogen als een hogere kwalitatieve afweer hebben gekregen terwijl de investering in structurele afweer en hergroei capaciteit zijn afgenomen (Tabel 1). Deze resultaten zijn in overeenstemming met de verwachtingen volgens de "Evolution of Increased Competitive Ability" hypothese en de "Shifting defense" hypothese. In verschillende andere invasieve plantensoorten vonden soortgelijke veranderingen plaats in allocatiepatroon (Liu and Stiling 2006; Doorduin and Vrieling 2011; Felker-Quinn et al. 2013). Tot nu toe was er echter onvoldoende bewijs voor de hypothese dat de veranderingen in herbivoren gemeenschap de verantwoordelijke selectieve kracht vormde aangezien de invloed van andere biotische en/of abiotische factoren niet uitgesloten kon worden (Willis and Blossey 1999; Colautti et al. 2004; Liu and Stiling 2006; Bradley et al. 2009; Colomer-Ventura et al. 2015). Dit proefschrift is een van de eerste onderzoeken waarin inheemse populaties met invasieve populaties van dezelfde plantensoort uit verschillende geografisch en klimatologisch gescheiden invasieve gebieden worden vergeleken. Door deze opzet kunnen de andere abiotische factoren mogelijk uitgesloten worden aangezien klimaatcondities worden gezien als de abiotische selectieve kracht met de meeste potentie voor evolutie na invasie (Bradley et al. 2009; Colomer-Ventura et al. 2015). Mijn resultaten toonden dat alle onderzochte eigenschappen consistent in dezelfde voorspelde richting veranderden in alle invasieve *J. vulgaris* populaties uit meerdere gebieden en niet gecorreleerd waren met veranderingen in klimatologische omstandigheden. Dit wijst er sterk op dat de afwezigheid van specialistische herbivoren de selectieve kracht was die geleid heeft naar de parallelle evolutionaire veranderingen van kenmerken in invasieve *J. vulgaris*. Of hetzelfde geldt voor andere invasieve plantensoorten valt nog te bezien. Om de rol van herbivoren in invasiviteit van plantensoorten beter te begrijpen moeten meer plantensoorten bestudeerd worden volgens dezelfde methode. Het begrijpen van de selectieve krachten achter de evolutionaire veranderingen in invasieve soorten kan helpen invasieve plagen te bestrijden.

Tabel 1 Samenvatting van de verschillen in eigenschappen tussen inheemse en invasieve Jacobaea vulgaris populaties. Waarden zijn gemiddelden ± SE, P waarden zijn van een genestte ANOVA, met herkomst (inheems versus invasief) als "fixed" factor, invasieve gebieden genest in herkomst en populaties genest in invasieve gebieden als "random" factoren. P (herkomst): significantie van genestte ANOVA tussen inheems en invasieve herkomst. NS= niet significant.

Hoofdstuk #	Eigenschap	Invasief	Inheems	*P* (Herkomst)
Hoofdstuk 2	Blad dikte (µm)	264.53±3.08	276.62±2.95	0.023
(17 weken groei)	Blad stugheid (kJ•m^{-2})	0.227±0.009	0.210±0.007	NS
	Blad massa per oppervlakte (g•m^{-2})	57.55±1.50	62.74±1.88	0.038
	Celwand eiwittenper oppervlakte (g•m^{-2})	9.48±0.31	10.63±0.25	0.037
	Totaal drooggewicht (g)	11.74±0.52	12.20±0.49	NS
	Wortel-spruit verhouding (g•g^{-1})	0.750±0.037	0.922±0.046	0.030
Hoofdstuk 3	Totaal drooggewicht (g)	3.73±0.14	3.33±0.11	0.041
(9 weken groei)	Specifiek bladoppervlak (cm^2•g^{-1})	238.09±2.14	228.3±2.48	0.044
	Fractie blad massa (g•g^{-1})	0.728±0.005	0.649±0.006	<0.001
	Pmax (µmol CO_2•m^{-2}•s^{-1})	23.13±0.43	21.48±0.38	0.005
	PNUE (µmol CO_2•g^{-1}•s^{-1})	18.86±0.44	17.02±0.41	0.011
	Totaal PA (µg•g^{-1} DW)	4144.6±152.8	2893.3±162.6	0.001
	Totaal PA (Tertiaire amines) (µg•g^{-1} DW)	1954.9±90.02	876.90±98.28	<0.001
	Totaal PA (N-oxide) (µg•g^{-1} DW)	2189.7±114.4	2016.4±104.8	NS
	Totale inuline inhoud in wortel (g)	0,397±0,025	0,597±0,028	<0.001
Hoofdstuk 4	Totaal drooggewicht op het moment van clipping (g)	2,14±0,09	1,90±0,08	NS(0,061)
(8 weken groei en 4 weken hergroei)	Totaal drooggewicht in de controle behandeling (g)	8,69±0,27	7,45±0,32	0,01
	Totaal drooggewicht na 4 weken hergroei (g)	1,58±0,06	1,97±0,09	0,003
	hoeveelheid inuline in wortel op het moment van clipping (g)	0,209±19,84	0,339±22,64	<0,001
Hoofdstuk 5	Spruit drooggewicht (g)			
(8 weken groei, 12 dagen herbivorie en 4 weken hergroei)	Na herbivorie door specialist	1.31±0.07	1.41±0.09	NS
	Na herbivorie door generalist	2.18±0.10	1.23±0.07	<0.001
	Zonder herbivorie	2.34±0.09	1.63±0.08	<0.001
	Spruit drooggewicht na hergroei (g)			
	Na herbivorie door specialist	1.90±0.20	2.49±0.15	0.037
	Na herbivorie door generalist	3.22±0.26	2.27±0.18	0.010
	Zonder herbivorie	5.16±0.42	3.31±0.28	0.002

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Tiantian Lin

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Leiden, the Netherlands

Curriculum Vitae

Tiantian Lin was born on 24th December, 1986, in Chengdu, Sichuan province, China. In 2005 she was admitted to Sichuan Agricultural University for her Bachelor of Science study in Garden and Landscape at the Faculty of Forestry. After four years of study, she finished her undergraduate research project "The effect of cadmium stress on morphological and physiological development of poplar plants" and received her BSc. degree in June 2009.

After that she continued her Master of Science study in Forest Cultivation at the same faculty with the project "The detoxification effect of nitrogen supplement on cadmium stress in poplar plants". After two years she completed her study and received her MSc. degree in September 2011.

In May 2011 she was granted a scholarship by the China Scholarship Council of the Chinese Ministry of Education for a four-year PhD study at Leiden University. In September, 2011 she arrived at Leiden and started her PhD study at the section of Plant Ecology and Phytochemistry at the Institute of Biology (IBL). During four years of study in Leiden she finished seven experiments and resulted in two publications, three submitted articles and this thesis.

Publication list

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- The 6th European Plant Science Retreat Workshop, July 2, 2014, Amsterdam, The Netherlands (Poster presentation).
- The 9th Plant-Insect Interactions Workshop, November 3, 2014, Utrecht, The Netherlands. (Oral presentation).
- The IBL Symposium 2014: Integrative Biology towards Healthy Communities, December 8, 2014, Leiden, The Netherlands. (Oral presentation).
- The 15th International Symposium on Insect-Plant Relationships (SIP), August 17-22, 2014, Neuchâtel, Switzerland. (Oral presentation).
- The 2015 Netherlands Annual Ecology Meeting (NERN), February 10 & 11, 2015, Wageningen, The Netherlands. (Oral presentation).
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